CHAPTER 6

A SIMPLE METHODOLOGY FOR HCV GENOTYPING AND RELATED INSIGHTS INTO "FATE" AND "RATE" AT THE SINGLE-MOLECULE LEVEL

6.1 The need for simple and easy HCV genotyping assay in limited-resource settings and an overview of existing technologies

HCV is a major public health concern and a leading cause of chronic liver disease and cirrhosis. An estimated population of 180 million are infected with HCV worldwide, and more than 350,000 people die every year from HCV-related liver diseases[45]. There is currently no vaccine for hepatitis C; however, it can be cured using antiviral medicines. Different genotypes respond differently to the same treatment; therefore, determining genotypes or even subtypes before treatment is necessary to adjust the dose and duration of the therapy, and predict the likely outcome. 7 genotypes of HCV are categorized, based on sequence similarity. Genotypes differ from one another by 31-33% at the nucleotide level. The diversity of sequence is not uniform in all regions of the HCV genome—some regions, such as 5'UTR, are quite conserved while other regions, such as E2, are hypervariable. Genotyping is necessary not only for the current standard therapy with PEG-interferon and ribavirin[46], but also for the new small-molecule antiviral drugs such as Sofosbuvir (Gilead)[47].

There are several HCV genotyping assays commercially available including sequencing analysis, real-time Taqman RT-PCR assays, and hybridization-based assays. Sequencing and phylogenetic analysis, such as Siemens TRUGENE HCV Genotype Test, give very accurate sequence information. However, performing sequencing is always time consuming, labor intensive and relatively expensive. Real-time Taqman RT-PCR assays, such as the Abbott RealTime HCV Genotype II assay, require only one RT-PCR step with fluorescent-labeled probes, but face the disadvantages of high initial setup cost and required instrument for real-time monitoring.

Hybridization-based assays, such as Siemens VERSANT HCV Genotype 2.0 assay (LiPA), are the most widely used HCV genotyping method. While they provides more subtype information than real-time PCR Taqman assay, the requirement for stringent control of conditions, a non-negligible degree of error due to secondary structures of the product, and different tolerance to single-nucleotide polymorphisms decrease their robustness. As pointed out, none of the methods discussed above are easily adoptable in limited-resource settings with a simple setup.

6.2 A novel way to use competition—the combination of RT-LAMP and restriction endonuclease to generate sequence-specific signal

Herein we introduced a new method for HCV genotyping based on the combination of isothermal amplification and sequence-specific inhibition. RT-LAMP was selected as the amplification chemistry because of its high tolerance to biological samples and compatibility with simple imaging devices[41a]. Restriction enzymes were chosen as the inhibitor considering their high specificity to DNA sequences under isothermal conditions. The underlying principle is that the inhibition takes place during amplification and the result of amplification itself serves as the readout.

We first retrieved 1398 sequences of HCV RNA from Los Alamos National Lab. All sequences were aligned using Genious software and the consensus of 5'UTR for each genotype was extracted based on the alignment result. RT-LAMP primers targeting 5'UTR were chosen from previous publication[48] and optimized to adapt to all genotypes. Based on the consensus, three restriction endonucleases were chosen to target the sequence differences between these four genotypes within the RT-LAMP amplicon. NheI (targeting GCTAGC) should recognize genotypes 1, 2, and 4; BsrBI (targeting CCGCTC) should recognize genotypes 1, 3, and 4; and BstNI (targeting CCWGG) should recognize only GT1 (Figure 17). Because one restriction endonuclease can probe multiple genotypes, in principle, an unambiguous genotyping panel can

be designed with fewer reactions than genotypes (e.g., three restriction endonucleases to differentiate four genotypes here).

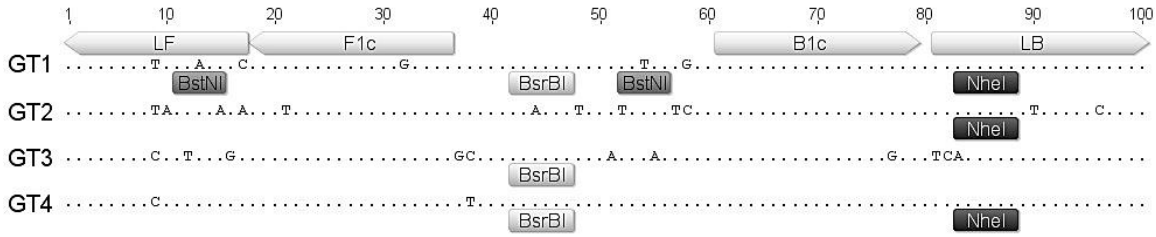

Figure 17 Sequence alignment of the consensus of four genotypes, shown together with the digestion sites of NheI, BsrBI, and BstNI. Dots stand for nucleotides that are the same between genotypes and the SNPs are shown with corresponding letters. LOOP_F (LF), F1c, B1c and LOOP_B (LB) are the priming regions. GT: genotype.

We first performed a real-time bulk measurement of different combinations of genotypes and restriction endonucleases. We obtained HCV isolates of genotypes 1, 2, 3, and 4 from commercial sources and sequenced the purified RNA to confirm the genotype assignment. For each genotype, we performed four experiments: one positive control without restriction endonuclease, and three experiments with one restriction endonuclease each. The experimental results (Figure 18B) agreed with the inhibition pattern predicted (Figure 18A): amplification of GT1 was inhibited by all three restriction endonucleases; amplification of GT2 was inhibited by NheI only; amplification of GT3 was inhibited by BsrBI; and amplification of GT4 was inhibited by NheI and BsrBI. Reactions with restriction endonucleases specific to the genotype were all delayed by a certain amount of time, although not fully inhibited. We then wished to further explore the reason for this delay: does the addition of the restriction enzyme simply slow down the reaction for each molecule, or does it decrease the number of molecules amplified?

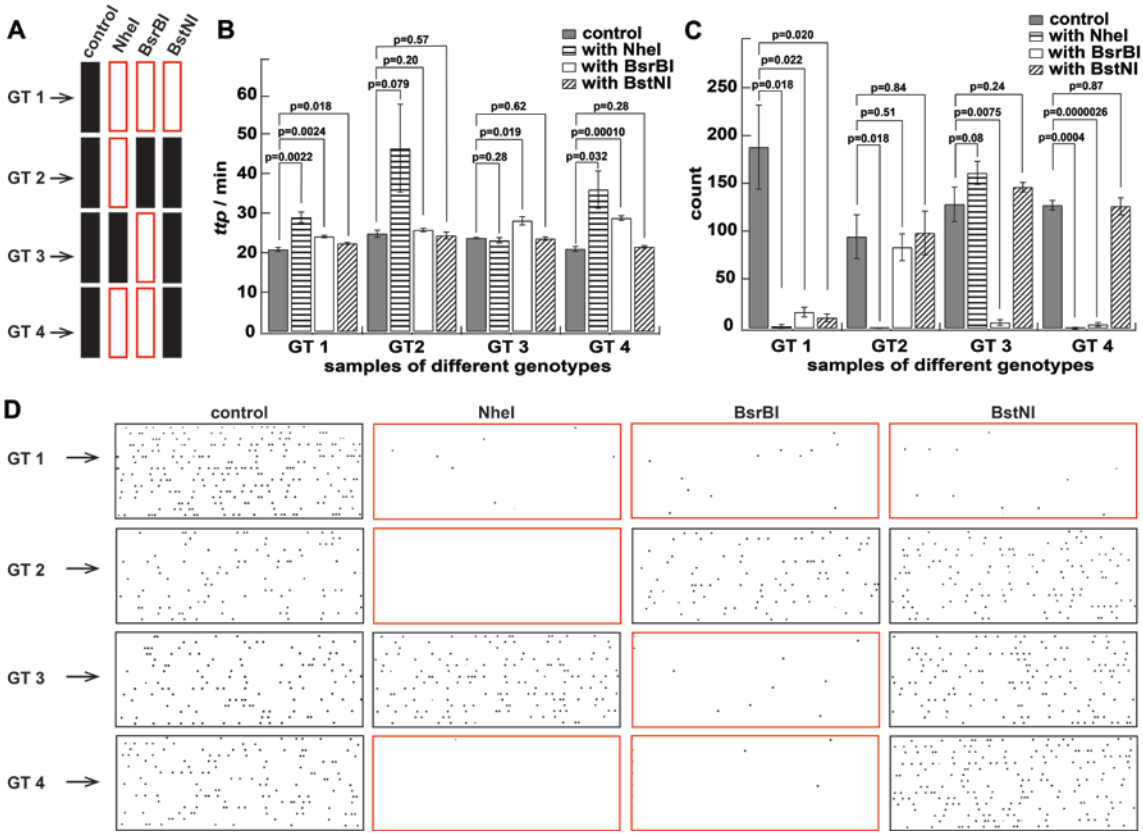

Figure 18 Predicted and experimental HCV genotyping pattern. (A) Prediction matrix for different combination of genotypes and restriction endonucleases. Red indicates predicted inhibition, black stands for normal amplification, and white stands for inhibition. (B) and (C) Graphs showing a comparison of HCV genotyping results using (B) real-time bulk assay (ttp: time-to-positive) and (C) end-point digital assay (n=3). (D) Photographs (inverted intensity) of end-point digital experimental genotyping results. The first column in both sections represents the positive control in the absence of restriction endonuclease and the following three columns indicate experiments with different restriction endonucleases. Each row represents a genotype of HCV RNA. Reproduced from the accepted version of reference 49. Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA

6.3 Improving assay performance when switching to a digital format

One way to answer the question above is to perform the assay in a digital format. We hypothesized that the count in digital format should be a direct measure of the amount of amplified molecules. In the digital format (Figure 18C and D), reactions with restriction endonuclease specific to the genotype showed reduced counts by at least 10 fold with a maximum p-value of 0.022 for all the combinations, indicating that the number of amplified molecules decreased by about 10 fold. Compared to a 10-100% change in bulk time-to-positive, this 10 fold

change significantly improved assay sensitivity. The activity and specificity of restriction endonuclease under LAMP conditions was further confirmed by DNA gel electrophoresis analysis of the digestion product. All four genotypes of HCV RNA were amplified with RT-LAMP before subjected to digestion with NheI-HF, BsrBI, and BstNI at RT-LAMP conditions (63 °C in LAMP buffer). The restriction pattern (Figure 19) matched the one predicted by informatics analysis.

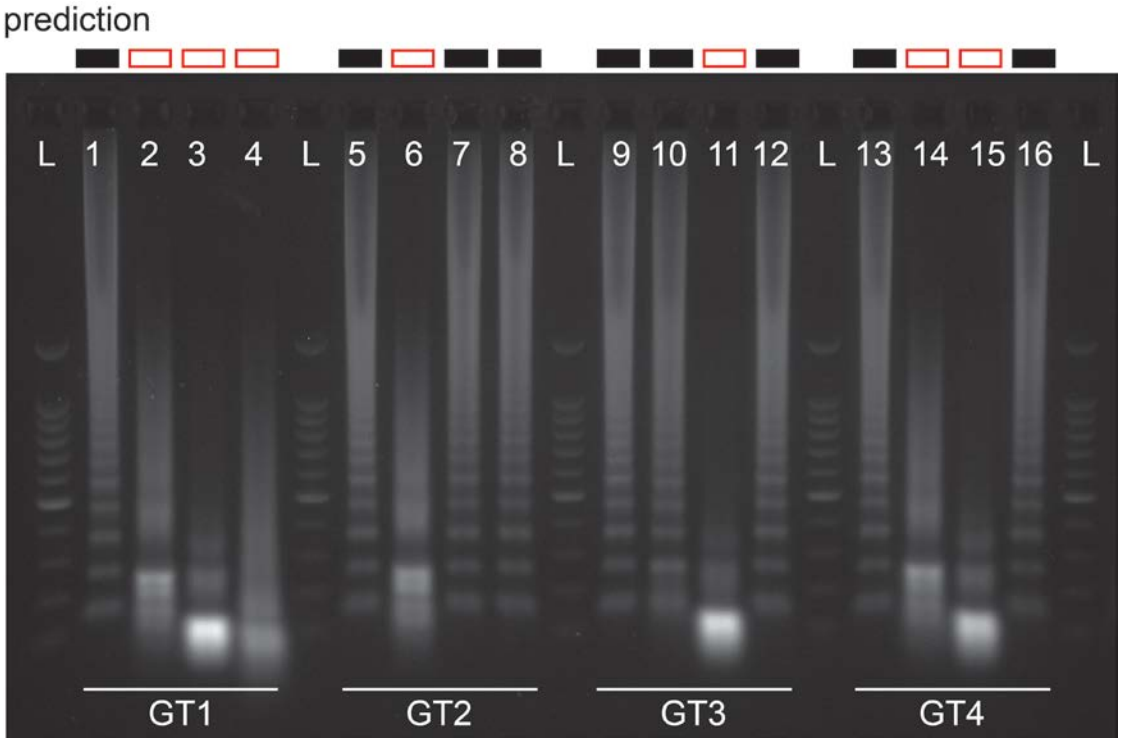

Figure 19 Image of DNA gel electrophoresis results for digestion of RT-LAMP product at RT-LAMP conditions. Lanes labeled with L are 100 bp DNA ladders. Lanes 1-4 are positive control, NheI digestion product, BsrBI digestion product, and BstNI digestion product for genotype 1, respectively. Lanes 5-8 are positive control and 3 RE digestion products for genotype 2. Lanes 9-12 are for genotype 3 and Lanes 13-16 for genotype 4. The specificity of RE to different genotypes are the same as predicted: for genotype 1 all 3 REs digested the product; for genotype 2 only NheI digested the product; for genotype 3 only BsrBI digested the product, and for genotype 4 both NheI and BsrBI digested the product. Reproduced from the accepted version of reference 49.

Both of these simplified measurements could in principle be used to derive genotype information from the competition between amplification and digestion. The advantage of the real-time

methodology is that it is well-established and the instrument already exists in many research labs. We, however, prefer the end-point digital format for limited-resource settings: it does not require complex instrumentation for performing kinetic measurements; it is expected to be robust to fluctuation in conditions; and it can be read-out with a cell phone, as stated in the published paper[49]. The viral load can be directly read from the positive control as well, giving more information for making clinical decisions. However, we do want to point out that the absolute measurement is not a necessity, because only the "extent" to which the digital counts decrease needs to be measured. The effectiveness of the inhibition chemistry determines the resolution of the digital measurement, with stronger inhibition resulting in lower requirements for resolution.

6.4 The capability of digital to decouple "fate" from "rate" by generating 2D data

To facilitate the discussion, here we use the same definition as in the published paper[49]: "rate" as how quickly amplification proceeds from a single molecule to produce a particular level of a signal and "fate" as whether or not amplification ultimately succeeds to provide that level of signal from a single molecule (Figure 20A). In the actual reaction scheme (Figure 20B), the fate-determining step occurs once the first double-stranded DNA (dsDNA) is formed (structure (1) in Figure 20B): either restriction endonuclease cleaves it, or it escapes the digestion and leads to the formation of the double-looped template that can be further amplified. Net rate of the overall reaction is determined by the rate of amplification and the rate of digestion, and should be the difference between the two.

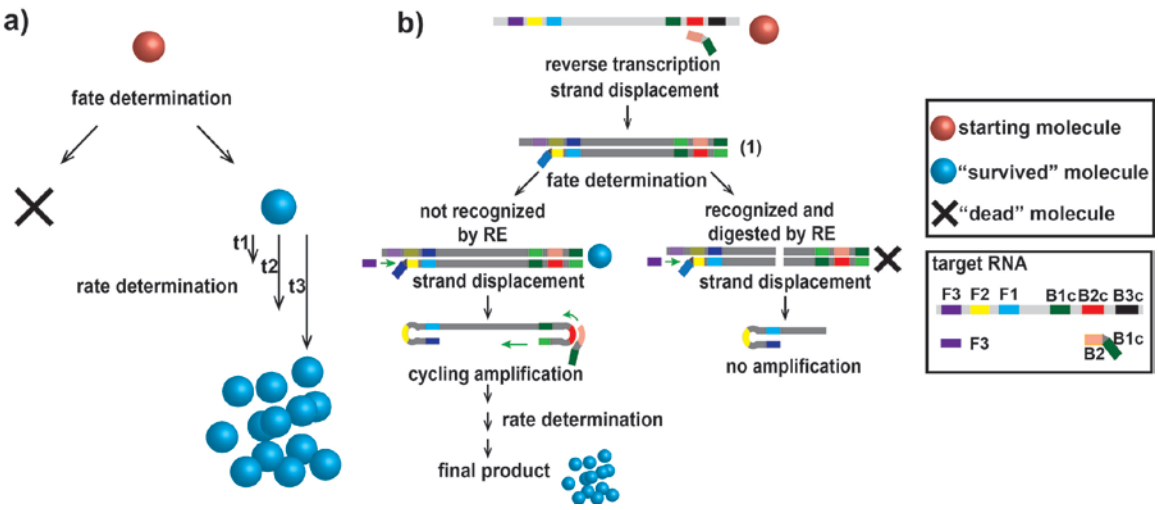

Figure 20 Schematic overview of the definition of "fate" and "rate". (A) In general, "fate" determines whether a molecule can proceed to the final stage of a reaction and "rate" determines how quickly the accumulation of products amplified from one "survived" molecule is. (B) For the competition between RT-LAMP and restriction digestion, "fate" determines whether the first dsDNA gets digested or proceeds to the cycling amplification and "rate" determines how quickly the amplicons accumulate. Reproduced from the accepted version of reference 49. Copyright © 2014 WILEY-VCH Verlag GmbH & Co. KGaA

To validate the hypothesis above, we performed real-time digital RT-LAMP/restriction endonuclease experiments with HCV genotype 1 RNA and BsrBI. The digestion site of BsrBI (CCGCTC) exists in the amplicon of GT1 RNA and therefore should inhibit the amplification. We used a SlipChip microfluidic device modified based on previous publication[42] to compartmentalize the reaction mixture and monitored the progress of amplification for each single compartment. We found significant heterogeneity of rate between molecules and a dramatic change in fate and smaller change in rate of target molecules (shift of mean in Figure 21A and B), indicating that in digital RT-LAMP, BsrBI affects fate more than it affects rate. The median of each set of real-time digital data (23 min for reaction without restriction endonuclease and 30 min for reaction with restriction endonuclease) were quite close to the mean shown in Figure 21A and B, indicating that there were not any data outliers that influenced the mean significantly (in other words, the distribution was close to an symmetric distribution) and that mean could be used to describe the distribution of time-to-positive in this case.

As a comparison, we performed the same competition experiments in the bulk real-time format using an RNA concentration equivalent to that of a single molecule in a well. Without BsrBI, the time-to-positive in this bulk experiment was close to that of the first positive molecule in the digital format (approximately 2 min slower) (Figure 21A). Upon addition of BsrBI, the bulk reaction showed increased variance and slowed down to the same extent as that for the first molecule in the digital format (Figure 21C). These data suggest that once exponential amplification of one molecule initiates, this process dominates the reaction mixture and is not affected by the amplification of the molecules that amplify later. The real-time digital format describes not only the fate, but also the rate of each molecule; thus, it provides more information than bulk real-time, which only reflects the rate of the first few amplified molecules, and end-point digital, which only reflects the fate.

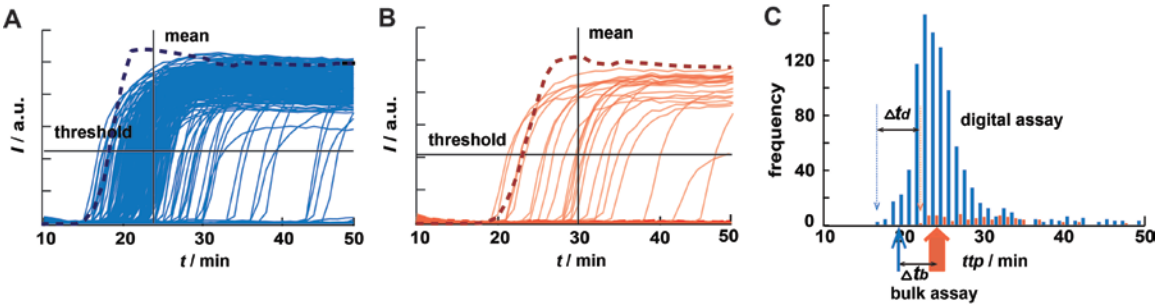

Figure 21 Results of real-time, single-molecule digital RT-LAMP/restriction endonuclease experiments for HCV RNA. (A), (B) Graphs showing 1280 fluorescence traces for the RT-LAMP amplification process of all the wells on a SlipChip device (solid light blue) and averaged fluorescence curve in bulk (dashed dark blue) in the absence of restriction endonuclease (A) and the traces for digital (solid light red) and for bulk (dashed dark red) in the presence of restriction endonuclease BsrBI (B). Horizontal solid lines indicate the threshold levels to consider a well positive. Vertical solid lines show the mean of the time-to-positive distribution. The scales in (A) and (B) are the same. (C) Graph showing the histogram of time-to-positive distribution for wells exceeding the threshold in (A), blue, and (B), red. The two bars below the x-axis show time-to-positive for real-time bulk experiments, the widths of which stand for standard deviation for the bulk assay (n=5). Reproduced from the accepted version of reference 49.

6.5 The connection between single molecule kinetics and bulk kinetics

The next question we asked is how to establish correlation between single molecule kinetics and bulk kinetics based on these findings. As we have discussed previously, bulk kinetics is only determined by the rate of first few amplified molecules. For the sake of simplicity, here we consider the bulk rate as only related to the rate of the first amplified molecule. The effect of various events (adding inhibitors, changing input target concentrations, changing temperatures, etc.) can all be traced to changes in the rate of the first amplified molecule.

In the case of adding restriction endonuclease, the entire distribution is shifted to the right (Figure 22A), which could be captured as the shift of the mean of the distribution from m1 to m2. As a result of this rate change, the first amplified molecule should have a later time-to-positive. Meanwhile, the fate of amplification is changed as well; hence each molecule has a smaller chance of being amplified, presented as the decrease of total counts (area under the peak). As a result, the probability of having molecules amplified at early time points is smaller. The change of time-to-positive from t1 to t2 for the first amplified molecule is a joint effect of both rate change and fate change, and therefore the difference between t1 and t2 (fate and rate effect) should be larger than that between m1 and m2 (pure rate effect). However, if the reaction scheme of amplification changes significantly, which changes the width of distribution, other considerations need to be taken into account to determine the time-to-positive for the first amplified molecule.

The same explanation applies to the case where the amount of input molecules increases (c1 to c2 in Figure 22B). The mean of the distribution will not change because the rate for each molecule is not influenced by the number of total molecules if the reagents are all in excess. The total number of counts and the frequency at each time point, though, will increase. This change of total number of molecules increases the probability of having molecules amplified at an early time point, which shifts the time-to-positive for the first molecule from t1 to t2 (Figure 22B), and causes a bulk time-to-positive decrease. When the concentration goes high enough, increasing number of

input molecules will not further shift the time-to-positive for the first molecule because it has reached the highest possible rate (determined by reaction condition). Further increases in the number of molecules will increase the frequency at this minimum time-to-positive (c2 to c3 in Figure 22B) and cause the bulk time-to-positive to decrease because of higher starting concentration.

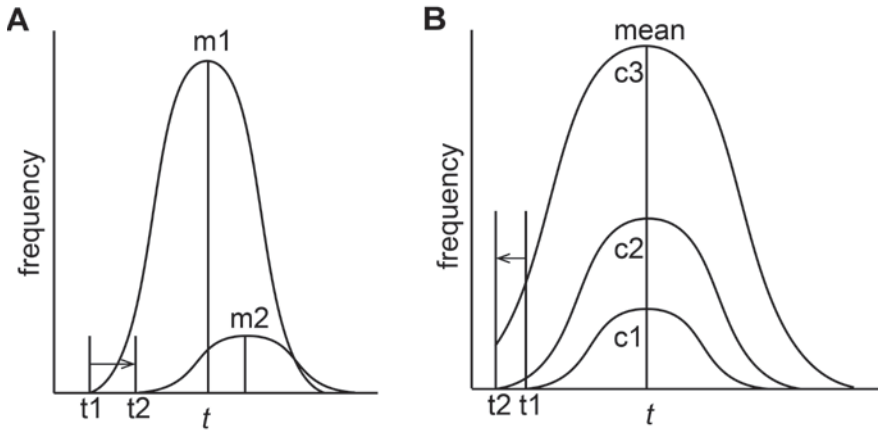

Figure 22 Theoretical histogram of time-to-positive distribution for single molecules (A) in the absence and presence of restriction endonuclease and B) at different input target concentration.

Now we can add some complexity, by discussing when the assumption that the bulk time-to-positive is only related to the first positive molecule holds true. Here a factor to consider is how the reaction progresses from one molecule to the final products in bulk: are all the products generated from one molecule, or from multiple molecules that start to amplify shortly after the first one does. If the amplification from a single molecule is fast enough, in the next minute (or any time frame), it generates more products than the number of molecules that start to amplify; therefore, the amplification in the entire tube is dominated by this one molecule. In this case, the difference between bulk time-to-positive and digital time-to-positive for the first molecule is the difference between time required to generate a positive signal in a 10μL solution in tube and that to generate a signal in a 3nL well. If the amplification from one molecule is slow, in the next minute the number of molecules starting to amplify will exceed the amount of products generated

by that first molecule; therefore the bulk kinetics are determined by multiple molecules starting to amplify at early time points. The difference between bulk time-to-positive and digital time-to-positive for the first molecule is the amount of time required for a certain amount of molecules to start amplification.

More questions raised by the results presented here await to be answered, as stated in the published paper[49]: "What is the right theoretical framework within which to analyze both rate and fate in single-molecule competition reactions? What are the molecular details of the mechanisms responsible for fate and rate determination in such systems? Can robustness of output of these systems be predicted *a priori*? What are the best amplification and inhibition chemistries with which to implement such competition reactions for genotyping and other genetic analyses?"

6.6 Improving the assay coverage by employing another amplification chemistry

The LAMP reaction is performed at 60 °C-65 °C, which is higher than the optimal temperature for many restriction endonucleases. This reduces the number of restriction endonuclease candidates and limits the application of the methodology. In place of LAMP, other isothermal amplification methods performed at lower temperatures have been tested including RPA and NASBA. While restriction endonuclease did not preserve their activity in RPA conditions, specific and complete inhibition of NASBA amplification with restriction endonuclease has been tested to be successful (Table 1). Experimental results agreed well with predicted results for the enzymes tested. Expanding the number of restriction endonuclease grants higher coverage of typable sequences and reduces mistyped and untypable cases. Based on the sequence analysis of 1300 sequences downloaded from Los Alamos National Laboratory, by using a combination of 7 restriction endonuclease, 97.6% of the sequences belonging to genotype 1a, 1b, 2, 3a and 4 can be correctly genotyped, 2% of the sequence are untypable, and only 0.4% of the sequences are mistyped. Moreover, in the RT-LAMP system, *ab initio* synthesis of DNA by Bst DNA

polymerase in the presence of restriction endonuclease limits the amount of restriction endonuclease in the system, which could be the reason for incomplete inhibition in the bulk format. In the NASBA system, no obvious *ab initio* synthesis has been observed; therefore the amount of restriction endonuclease could be increased for better inhibition. A complete stop of the amplification reaction in the bulk format avoids the requirement for real-time monitoring, enabling direct end-point readout for bulk format.

Table 1 Predicted and experimental NASBA results for combinations of genotypes and restriction endonuclease

	NheI	BsrBI	ApoI	BsrGI	BseYI	BstXI	NruI
				Predicted results			
GT 1	–	–	+	+	+	+	–
GT 2	–	+	+	+	–	–	+
	–	+	+	+	+	+	+
	+	+	+	+	–	–	+
	+	+	+	+	+	+	+
GT 3	+	–	–	+	–	+/–*	+/–
GT 4	–	–	+	–	+	+	+/–
	–	–	–	+	+	+	+/–
	–	–	–	–	+	+	+/–
				Experimental results			
GT 1	–	–	+	+			
GT 2	n.t.**	+	+	+		n.t.	
GT 3	+	–	–	+			
GT 4	–	–	n.t.	n.t.			

* +/–: NASBA reaction could be positive or negative. Information extracted from this combination does not contribute to genotyping.
** n.t.: not tested

This research opens the door for sequence-specific recognition using competition reactions. Similar principles could be applied with other sequence-specific inhibitors such as peptide nucleic acid (PNA) and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 genome editing systems, other isothermal amplification chemistries, and in any applications such as rare allele detection and cancer diagnosis.