Multiplex Screening Kit for Routine Monitoring of HIV Suppression and Drug Resistance

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Disclosure: There are no conflicts of interest for this project

Background
Real-time PCR drug resistance assay development and use

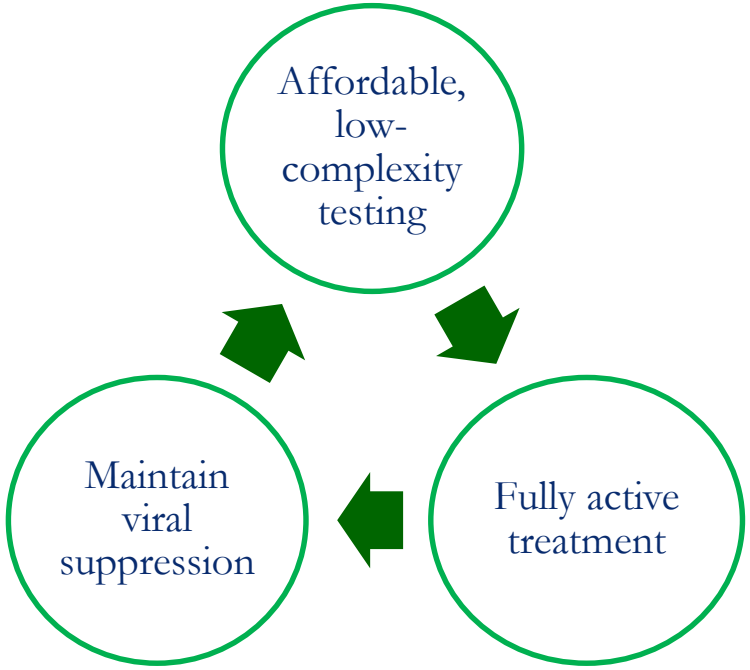
The Problem

- **Global clinical management of HIV infection is lagging because necessary testing is uncoupled, complex and often exceeds local resources**

Ending the HIV Epidemic: A Plan for America

- Diagnose all people with HIV as early as possible after infection
- Treat the infection rapidly and effectively to achieve sustained viral suppression.
- Protect people at risk for HIV using potent and proven prevention interventions, including PrEP, a medication that can prevent HIV infections.

- **The second goal is the most daunting, partly impacted by emergence and spread of drug resistance**
- **Prevention efforts have reached a plateau due to inadequate testing**
- **Treatment options subject to cost and availability**

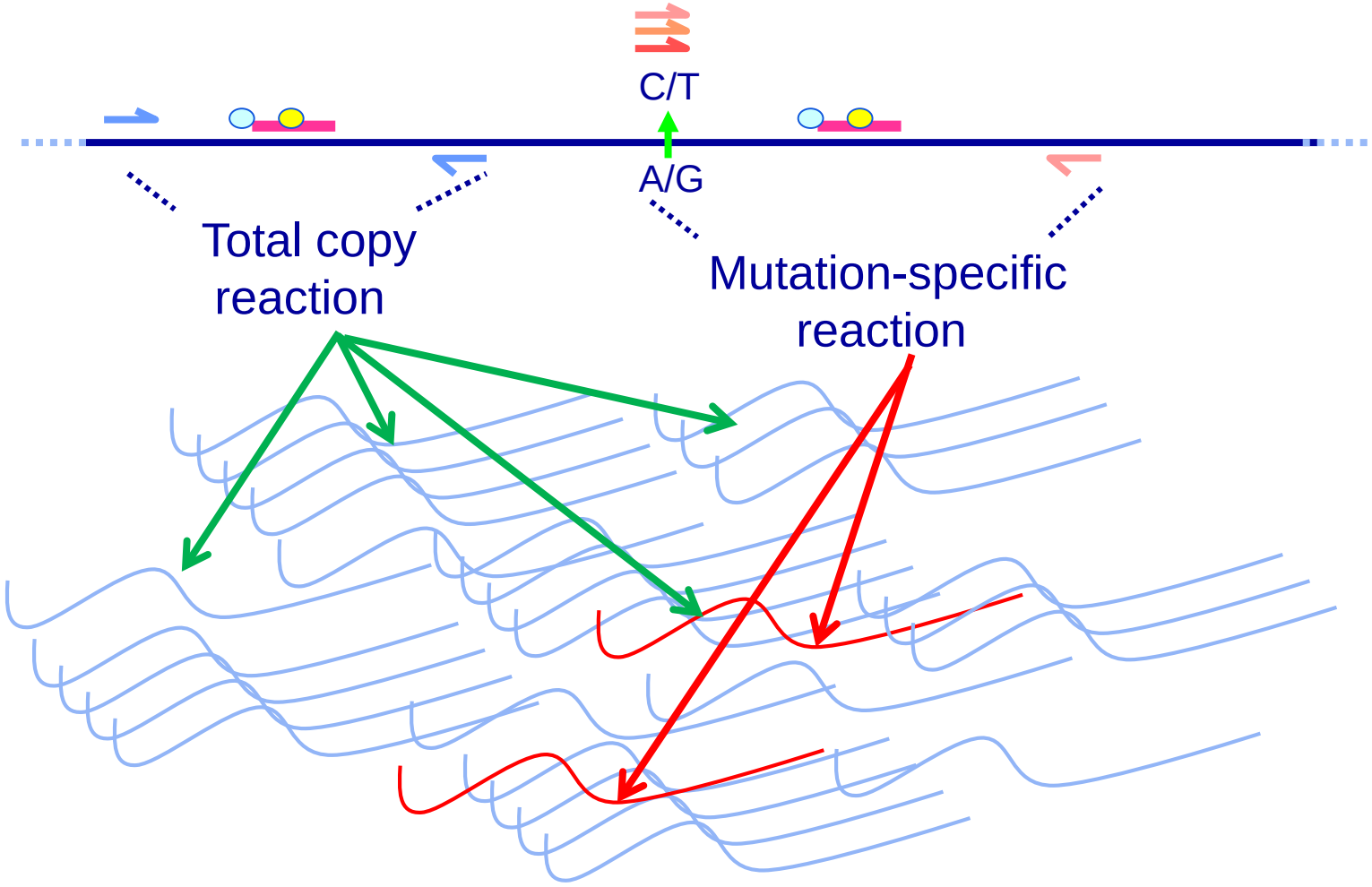


Simple and cost-effective assays that *combine virus load and drug resistance measurements* for large-scale, routine use would benefit virus suppression and inform rational ART regimen switching

Background

Real-time PCR Clinical Screening Assay

History in detecting minority-level resistance



Background

AIDS 2016

Sensitive sentinel mutation screening reveals differential underestimation of transmitted HIV drug resistance among demographic groups

Jin-fen Li, Laurie Linley, Richard Kline, Rebecca Ziebell, Walid Heneine and Jeffrey A. Johnson

Table 2. Prevalence of majority and minority levels of five sentinel transmitted drug resistance mutations, among HIV-diagnosed persons from US surveillance areas, 2009–2011.

Mutation	Bulk genotype (%)	Sensitive PCR screening (%)	% increase
M41L	12 (1.1)	15 (1.4)	30
K65R	0 (0)	18 (1.7)	>340*
K103N	75 (7.0)	90 (8.4)	20
Y181C	9 (0.8)	29 (2.7)	320*
M184V	3 (0.3)	15 (1.4)	500*
Cases of TDR	85 (7.9)	145 (13.6)*	70

*$P < 0.0001$, for K65R 0.5% is used as the bulk prevalence for logit estimation.

US surveillance specimens

High fitness cost mutations are significantly underestimated by conventional genotyping

The **HIV Multiplex Screening Assay** Allows Screening of Infected/At-Risk Persons at *Appropriate* Intervals

- HIV mutates rapidly and nucleic acid testing is needed to detect resistance mutations
- Years of R&D on sensitive assays for multiple HIV subtypes

  [Good for research – But complexity is bad for wide-scale patient management]
- For Point-of-Care, minimal infrastructure and training is best – Less is More

→ De-evolution of simplex real-time assays to a straight-forward multiplex format that measures:

1) If the person is **expressing** HIV, if so,
2) Is it due to infection with or emergence of a drug-resistant strain

Multiplex screening assay rationale

Development of Multiplex Design

- An antiretroviral regimen for a treatment-naive patient generally consists of two NRTIs in combination with a third active ARV drug from one of three drug classes INSTI, NNRTI, PI (https://aidsinfo.nih.gov/guidelines)
- For US domestic resistance screening we selected different mutation combinations for surveillance of transmitted resistance that are also commonly associated with current virologic failures.

Major Nucleoside RT Inhibitor (NRTI) Resistance Mutations

	Non-TAMs					TAMs						MDR	
	184	65	70	74	115	41	67	70	210	215	219	69	151
Cons	M	K	K	L	Y	M	D	K	L	T	K	T	Q
3TC	VI	R										Ins	M
FTC	VI	R										Ins	M
ABC	VI	R	E	VI	F	L			W	FY		Ins	M
TDF	***	R	E		F	L		R	W	FY		Ins	M
ZDV	***	***	*	*		L	N	R	W	FY	QE	Ins	M

Major Non-Nucleoside RT Inhibitor (NNRTI) Resistance Mutations

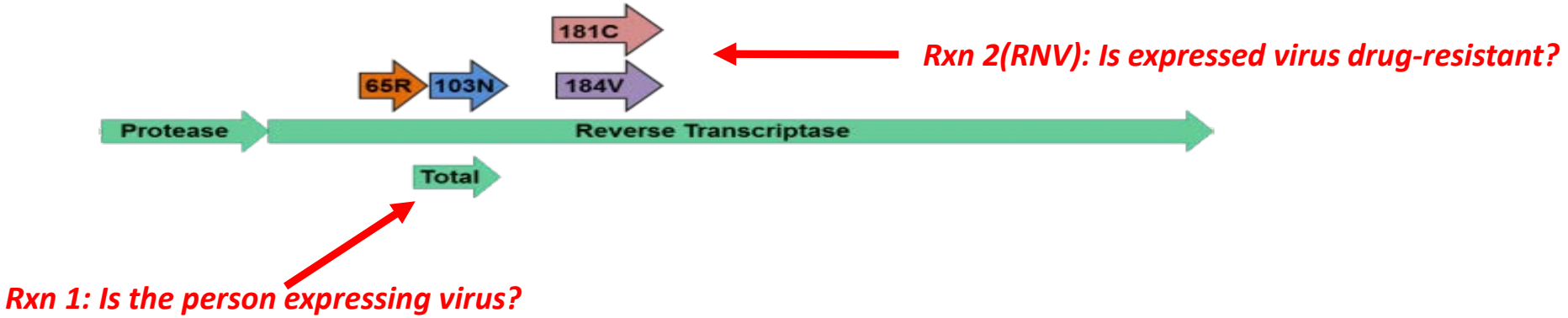
	100	101	103	106	181	188	190	230
Cons	L	K	K	V	Y	Y	G	M
NVP	I	EP	NS	AM	CIV	LCH	ASEQ	L
EFV	I	EP	NS	AM	CIV	LCH	ASEQ	L
ETR	I	EP			CIV	L	ASEQ	L
RPV	I	EP			CIV	L	ASEQ	L

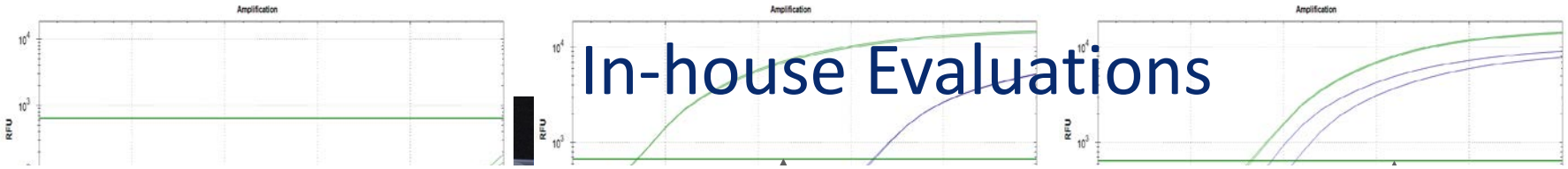
hivdb.stanford.edu

De-evolution of simplex real-time PCR assays

- We designed HIV pan-subtype multiplex screening assays to accommodate three to four simultaneous mutation-specific reactions, we settled on the most relevant for current first-line regimens
  - RNV assay detects RT *K65R*, K103N, *M184V* mutations; 65R and 184V are also important for PrEP
- The original assay amplicon lengths for each reaction were shortened to less than 200 bp to minimize background fluorescence and prevent interference from overlapping amplicons
- Specific FAM-quencher probes were replaced with a less-expensive intercalating dye

Multiplex Real-time PCR Screening Assay Design





In-house Evaluations

- Methods were evaluated against previously characterized subtype B (n=197), A (n=30), D (n=12), and C (n=39) clinical specimens
- The multiplex maintained specificity while losing some sensitivity with samples having targeted mutations at frequencies at <2%

Inexperienced Visiting Summer Science Student
Multiplex 3 & 4 Blinded testing clinical samples

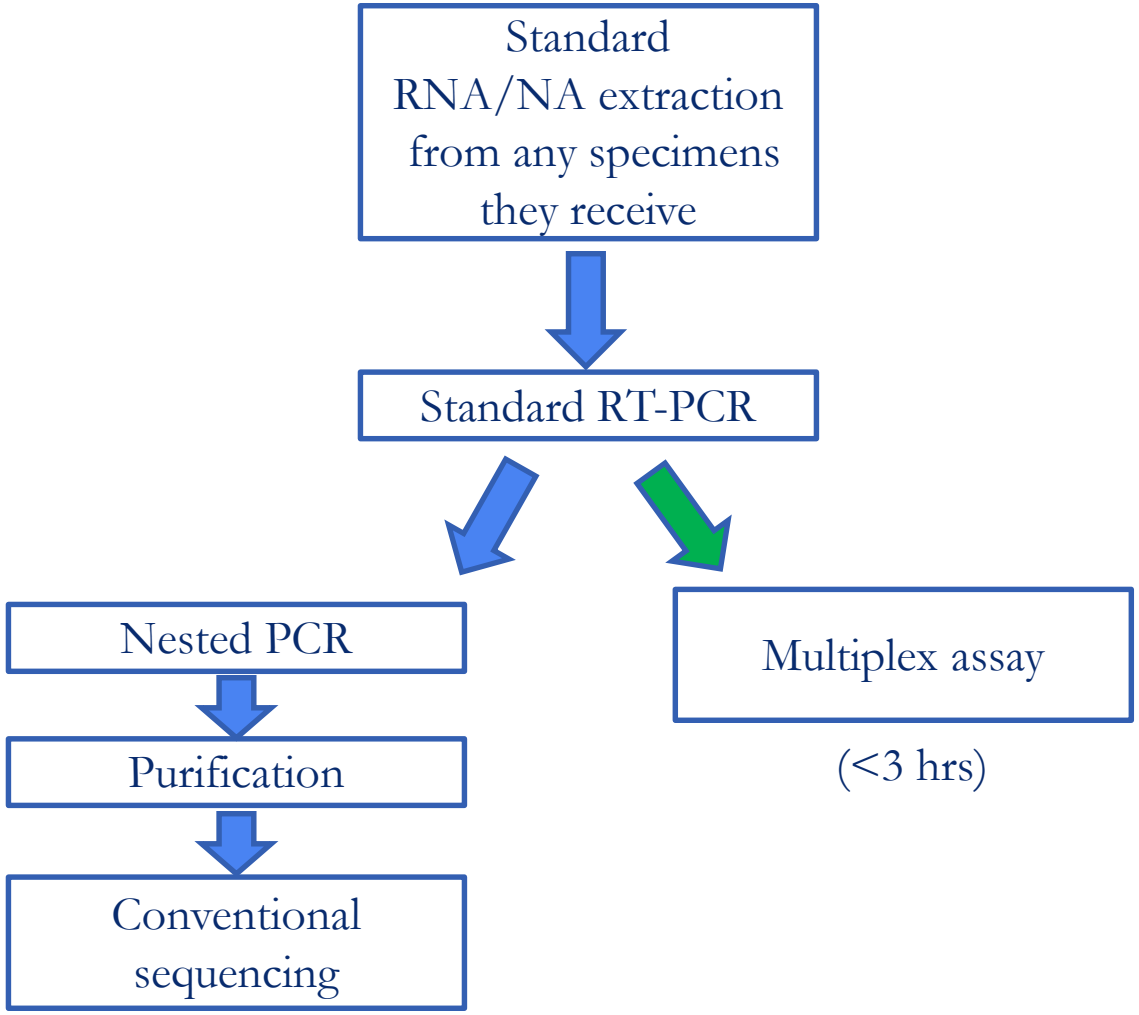
- Multiplex screening and analysis was done successfully with minimal training necessary
- All DR mutations previously detected by bulk genotyping were successfully detected by multiplex screening

	Multiplex Test		Simplex Real-Time Test			Sequence
ID	R/N/C	R/N/V	K103N	Y181C	M184V	Bulk 6wk
1	0.2	0.4	7.3	6.7	15.1	K103N
2	3.9	6	n/a	1.08	24.37	K103N, Y181C
3	5.3	6.1	3.6	3.7	14.6	K103N,Y181C
4	5.4	6.6	9.40	6.3	13.6	103N, 106V
5	6.4	7.7	7.4	4.2	25.4	K103N, Y181C
6	6.5	8.2	6.8	7.2	18	K103N
7	7.5	9.4	5.2	6.8	16.6	K103N, Y181C
8	7.5	6.8	6.1	23.1	18.7	K103N, Y181C
9	8.1	7.4	9.3	9	15.6	K103N, Y181C
10	8.4	9.1	8.5	9.9	11.5	K103N

	Multiplex Test		Simplex Real-Time Test			Sequence
ID	R/N/C	R/N/V	K103N	Y181C	M184V	Bulk 6wk
11	9.4	9.9	7.9	8.1	14.4	
12	9.8	9.3	15.6	7.3	14.9	
13	9.9	9.7	16.8	6.5	15.1	
14	11.3	11.2	16.5	13.8	17.9	
15	11.3	11	16.5	20.8	13.6	
16	12.3	10.5	14.6	15.1	17.8	
17	12.4	10.3	17.7	16.6	14.7	
18	12.8	10.2	13.6	9.7	14.1	
19	12.9	11.8	17.8	16.4	15	
20	13	12.5	16.6	21.6	19.9	

HIV Drug Resistance Multiplex Screening Kit Development

Assay uses site's in-house procedures



Lyophilize and QC-validate kits

- Produce a kit for ambient temperature transportation (no cold chain)
- We performed a pilot study of trehalose as a lyoprotectant at concentrations of 2%, 3%, 4% & 5% (w/v) – reconstituted reagents and tested each concentration for comparison to non-lyophilized reagents
- 4% trehalose (w/v) gave the most consistent results

Reagent Lyoprotectant Evaluation

(Mutant Positive Plasmid)

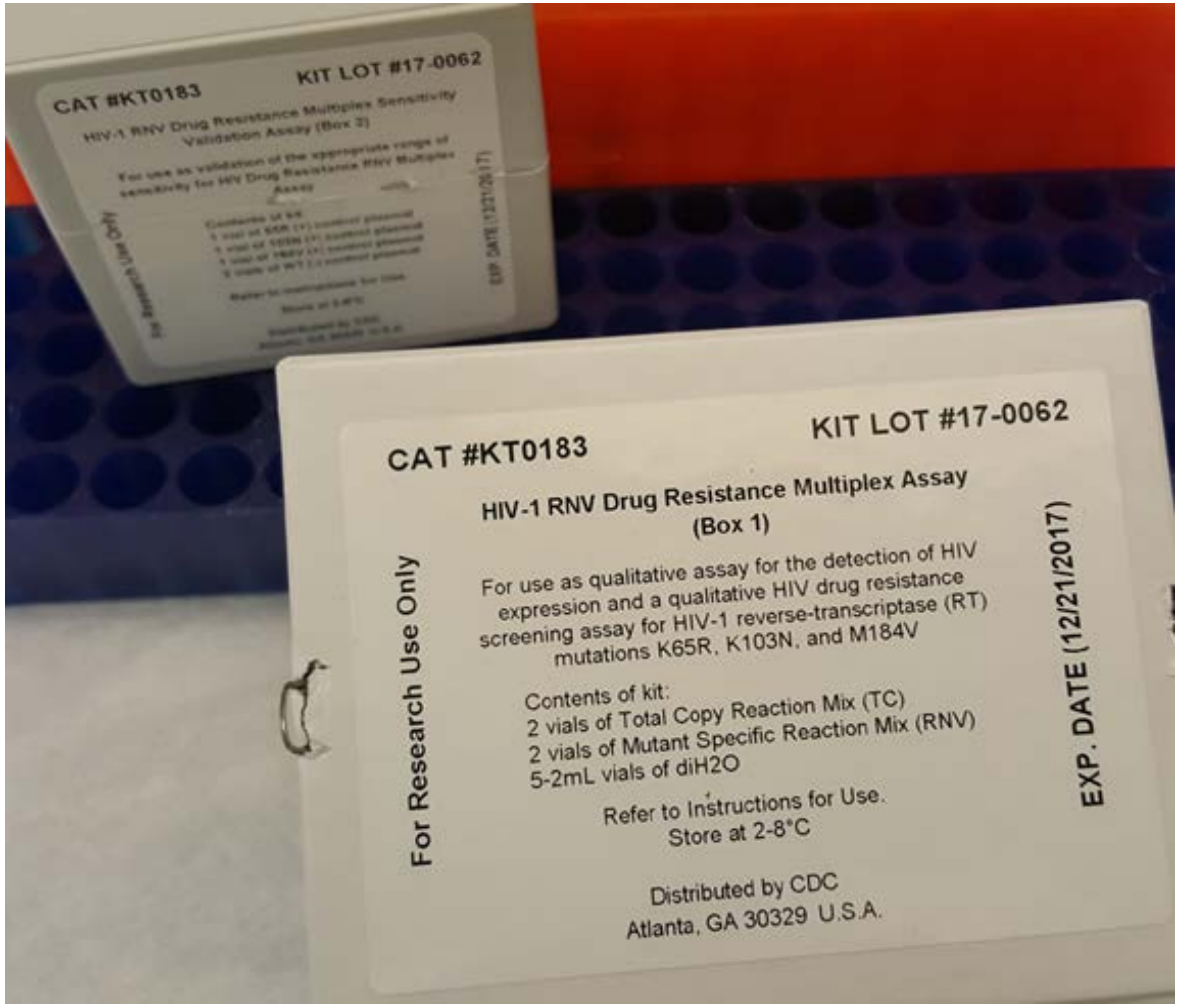
% Trehalose	ΔCT	RNV SD	TC SD
4%	3.89	0.162	0.056

Reagent Lyoprotectant Evaluation

(Wildtype Negative Plasmid)

% Trehalose	ΔCT	RNV SD	TC SD
4%	13.38	0.391	0.170

Production lot kit



Package labelling

Catalogue # XXXXXX
Lot # XX-XXXX
Expiration date: XX/XX/XXXX

HIV-1 RNV Drug Resistance MultiPlex Sensitivity Validation Assay

Purpose:

This assay is used to validate the range of sensitivity for the HIV RNV Drug Resistance Multiplex Assay on new instrumentation using positive control plasmids diluted into wildtype negative control plasmid background.

Kit Components:

2 vials of TC Control Sensitivity Mix (Blue)
2 vials of RNV Control Sensitivity Mix (Red)
1 vial of 65R(+) control plasmid
1 vial of 103N(+) control plasmid
1 vial of 184V(+) control plasmid
2 vials of WT(-) control plasmid (Purple)
10 mL of diH2O (Silver)

Starting Material:

Control plasmids

Catalogue # XXXXXX
Lot # XX-XXXX
Expiration date: XX/XX/XXXX

HIV-1 Drug Resistance RNV MultiPlex Assay

Purpose:

This assay is used as a qualitative assay for the detection of HIV expression and a qualitative HIV drug resistance screening assay for a panel of three key resistance mutations associated with drug resistance, HIV-1 reverse-transcriptase (RT) mutations K65R, K103N, and M184V. The assay principle is to detect relative amplification levels of a virus total copy (TC) PCR to a mutation-specific (RNV) PCR which are detected using an intercalating fluorescent dye on real-time PCR platforms. The total copy (TC) reaction serves as the qualitative measure of unsuppressed HIV. The TC reaction resulting in a Ct value of 1- 30 cycles is positive for the presence of unsuppressed HIV (lower limit of detection = 600 cps/mL)

Kit Components:

2 vials of Total Copy Reaction Mix (TC) (Blue)
2 vials of Mutant-Specific Reaction Mix (RNV) (Red)
1 vial of RNCV positive (+) control plasmid (Orange)
1 vial of WT negative (-) control plasmid (Purple)
10 mL of diH2O (Silver)

Starting Templates:

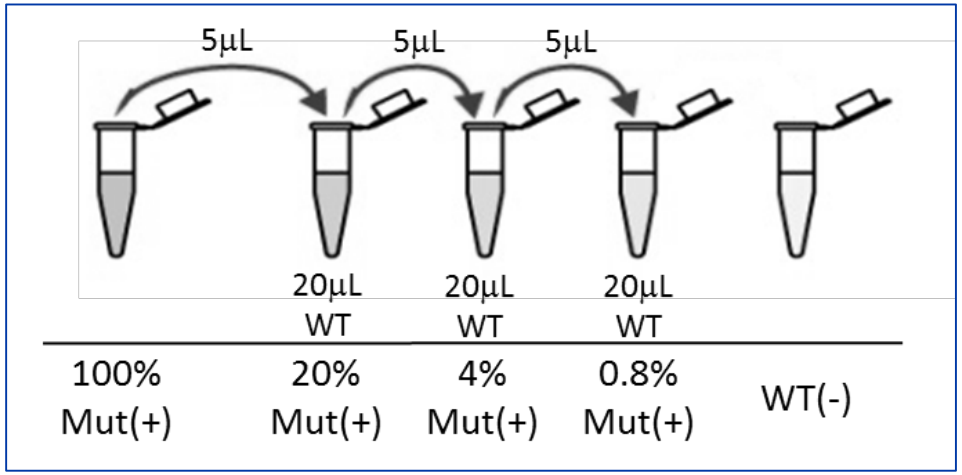
Reverse transcriptase-polymerase chain reaction (RT-PCR) products from test samples and control plasmids

HIV-1 Drug Resistance RNV MultiPlex Assay Purpose

- This assay is used as a qualitative assay for the detection of HIV expression and a qualitative HIV drug resistance screening assay for a panel of three key resistance mutations associated with drug resistance
- Targets HIV-1 reverse-transcriptase (RT) mutations : K65R, K103N, and M184V
- The assay principle is to detect relative amplification levels of a virus total copy (TC) PCR to a mutation-specific (RNV) PCR, which are detected using an intercalating fluorescent dye on real-time PCR platforms
- The total copy (TC) reaction serves as the qualitative measure of unsuppressed HIV (lower limit of detection = 600 cps/mL DBS, 250 cps/mL plasma)

HIV-1 RNV Drug Resistance MultiPlex Sensitivity Validation Assay

- For pre-shipment QC and site validation
- This assay is used to validate the range of sensitivity for the HIV RNV Drug Resistance Multiplex Assay on new instrumentation using positive control plasmids diluted into wildtype negative control plasmid background.



100% 65R TC	100% 65R TC	100% 65R TC	100% 65R RNV	100% 65R RNV	100% 65R RNV	20% 65R TC	20% 65R TC	20% 65R TC	20% 65R RNV	20% 65R RNV	20% 65R RNV
4% 65R TC	4% 65R TC	4% 65R TC	4% 65R RNV	4% 65R RNV	4% 65R RNV	0.8% 65R TC	0.8% 65R TC	0.8% 65R TC	0.8% 65R RNV	0.8% 65R RNV	0.8% 65R RNV
100% 103N TC	100% 103N TC	100% 103N TC	100% 103N RNV	100% 103N RNV	100% 103N RNV	20% 103N TC	20% 103N TC	20% 103N TC	20% 103N RNV	20% 103N RNV	20% 103N RNV
4% 103N TC	4% 103N TC	4% 103N TC	4% 103N RNV	4% 103N RNV	4% 103N RNV	0.8% 103N TC	0.8% 103N TC	0.8% 103N TC	0.8% 103N RNV	0.8% 103N RNV	0.8% 103N RNV
100% 184V TC	100% 184V TC	100% 184V TC	100% 184V RNV	100% 184V RNV	100% 184V RNV	20% 184V TC	20% 184V TC	20% 184V TC	20% 184V RNV	20% 184V RNV	20% 184V RNV
4% 184V TC	4% 184V TC	4% 184V TC	4% 184V RNV	4% 184V RNV	4% 184V RNV	0.8% 184V TC	0.8% 184V TC	0.8% 184V TC	0.8% 184V RNV	0.8% 184V RNV	0.8% 184V RNV
WT(-) TC	WT(-) TC	WT(-) TC	WT(-) RNV	WT(-) RNV	WT(-) RNV	H2O TC	H2O TC	H2O TC	H2O RNV	H2O RNV	H2O RNV

Validation Results Analysis Guidelines

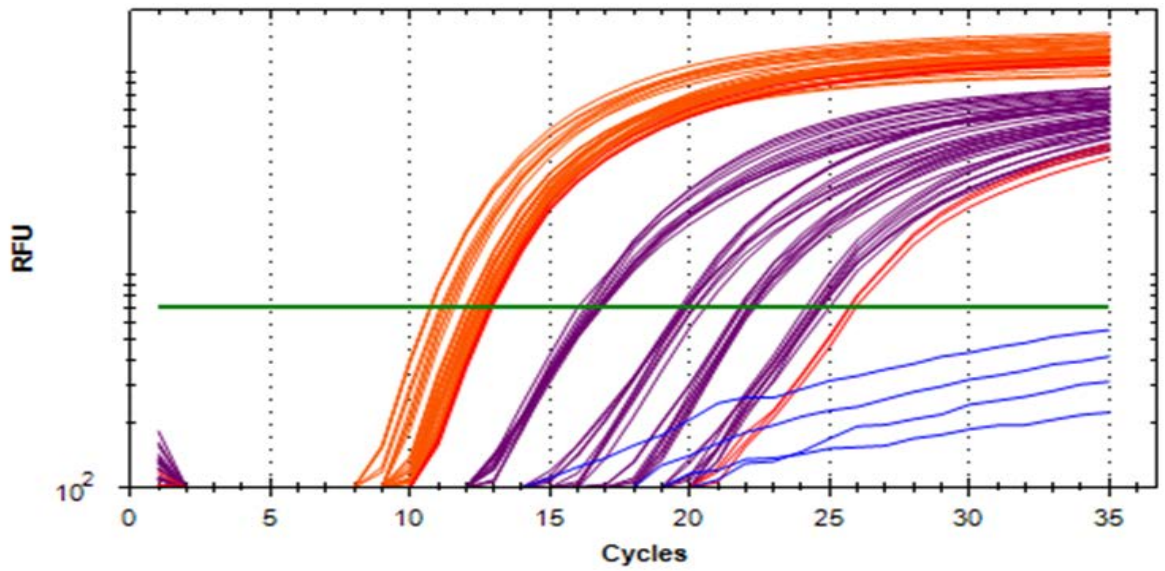
Control Sensitivity Results:

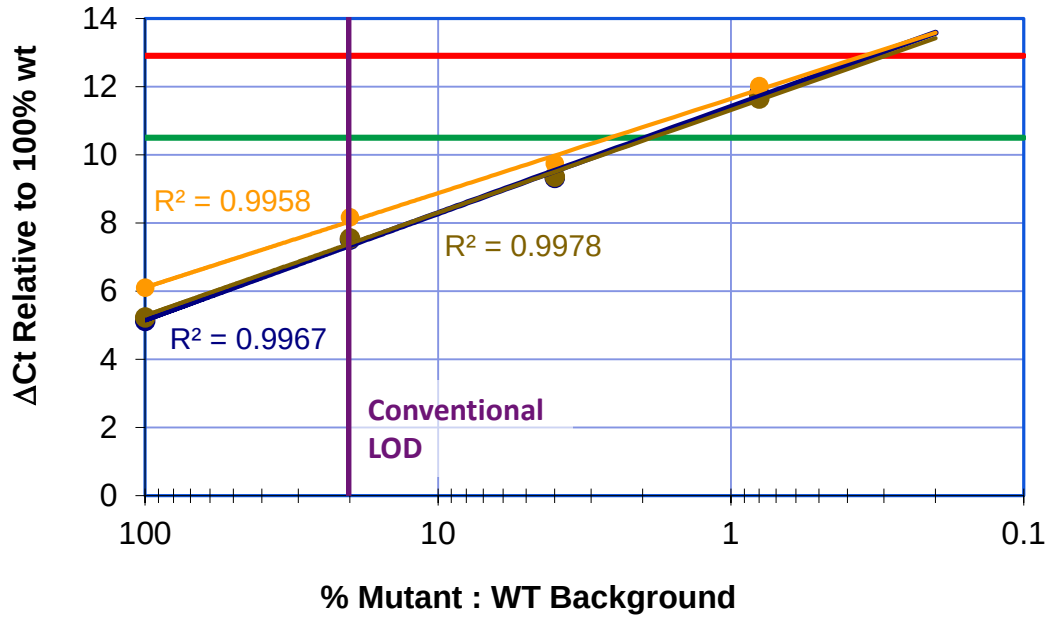
- Calculate mean ΔCt:

Mean ΔCt Calculation = RNV calculated mean Ct value – TC calculated mean Ct value

- The established ΔCt cutoff for positive detection for the presence of mutation is set at 10.5 cycles.
- The calculated mean ΔCt for 4% mutant plasmid in wildtype plasmid background should fall beneath the established cutoff of 10.5 cycles for successful kit validation

Pre-shipment Lot Quality Control Validation Check





% Mutant:WT Plasmid Background

	100%	20%	4%	0.80%	0% - WT
65R ΔCt	5.13	7.51	9.32	11.79	12.90
103N ΔCt	6.10	8.17	9.75	12.02	12.90
184V ΔCt	5.23	7.55	9.36	11.65	12.90

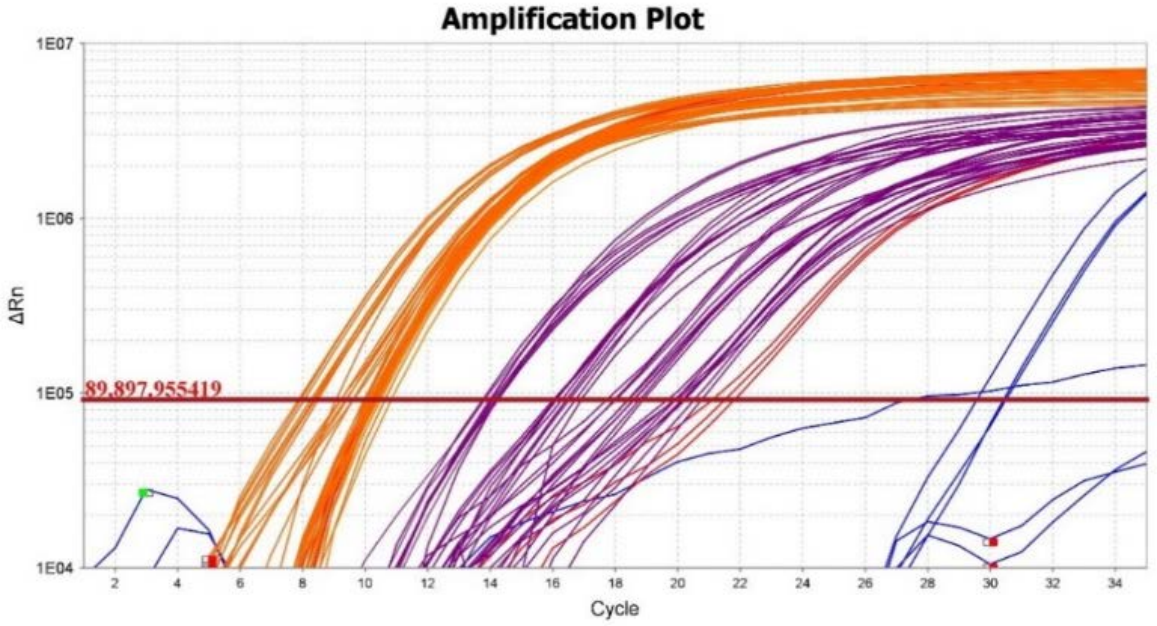
Standard Conditions for HIV-1 Drug Resistance RNV MultiPlex Screening Assay

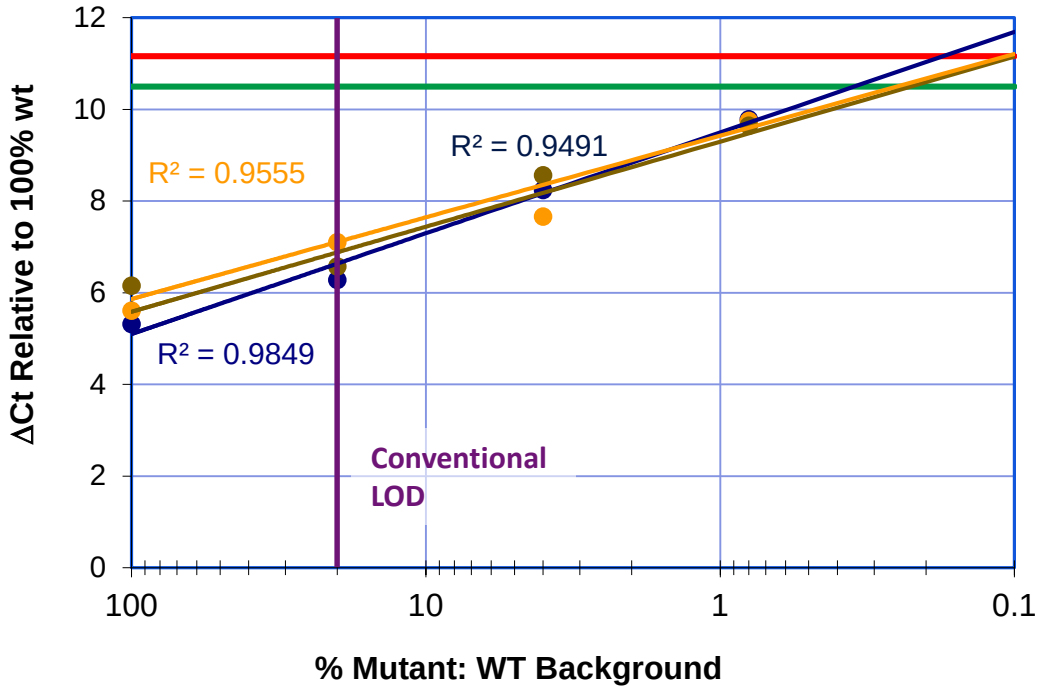
- For reliable results, the Ct value for the sample TC reaction should fall within 10 to 25 cycles
  - If the RT-PCR product is too concentrated it should be diluted in diH_2O to fall within this range
- ΔCt calculation = RNV Ct value – TC Ct value
- RNCV(+) control ΔCt is ≤5.5 PCR cycles
- WT(-) control ΔCt is ≥10.5 PCR cycles
- ΔCt cutoff for positive detection of mutation is ≤10.5 PCR cycles.

Full lot kit manufacturing

- Completed production of the first and second lots of 25 kits each to be sent to Jo'burg, South Africa and Kisumu, Kenya for evaluation
- Successfully completed the in-house QA/QC evaluation of first and second lot productions of 25 kits each - acceptable performance for lot distribution
- First lot successfully delivered to South Africa 07/20/2017
- Second lot successfully delivered to Kenya on 09/01/2017
- The sensitivity validation kit was used to establish on-site setup and validation for samples testing

On-site (Kisumu) Assay Validation Results





% Mutant:WT Plasmid Background

	100%	20%	4%	0.80%	0% - WT
65R ΔCt	5.31	6.28	8.24	9.78	11.16
103N ΔCt	6.15	6.57	8.56	9.64	11.16
184V ΔCt	5.61	7.1	7.66	9.75	11.16

Direct Comparison of Validation Runs

		% Mutant:WT Plasmid Background			
		100%	20%	4%	0.80%
65R ΔCt	CDC	5.13	7.51	9.32	11.79
	Site	5.31	6.28	8.24	9.78
103N ΔCt	CDC	6.10	8.17	9.75	12.02
	Site	6.15	6.57	8.56	9.64
184V ΔCt	CDC	5.23	7.55	9.36	11.65
	Site	5.61	7.10	7.66	9.75

QC target (≤ 10.5)

Ambient shipping international was a success

Public Health Impact

- This simpler approach to combined HIV testing can make monitoring HIV infection more feasible for overburdened/under-resourced HIV care systems
- The ability to frequently test and monitor suppression in disproportionately affected populations will change the trajectory of HIV incidence

➢ Frequent viremia and resistance screening can prevent:

1) costly switches to second-line therapies if there is no drug resistance,
2) accumulation of drug resistance in persons not fully suppressing,
3) secondary transmission of resistant variants, and
4) unnecessary use of already limited funds to genotype wildtype infections; thereby, focusing on persons who truly need resistance genotyping and making public health dollars go farther

Conclusions – Future Directions

- We present a simplified method to simultaneously assess viremia and DR that could support routine screening in resource-constrained and local HIV care systems
- The HIV-1 Drug Resistance RNV MultiPlex Assays can be easily established at field sites using readily available instrumentation
- For patient management, specimens that screen resistance-positive could be selected for complete sequencing
- Serve in initial and/or routine HIV monitoring for pre-exposure prophylaxis administration
- Interest in establishing domestic field site collaborations to evaluate the local utility of the assay
- Detection of other resistance mutations to current recommended treatment regimens (i.e., integrase inhibitor mutations) under consideration

Acknowledgments

HIV Drug Resistance Diagnostic Development Activity

- Jeff Johnson
- Ariana Santos
- Jin-fen Li
- Xierong Wei

CDC Diagnostic Manufacturing Team

- Kanwar Bedi
- Kaiser Bijli

AIDS Virus Research Unit

Centre for HIV and STIs

Johannesburg, South Africa

- Gillian Hunt
- Catherine James

KEMRI-CDC HIV-R Laboratory

Kisumu, Kenya

- Maxwell Majiwa
- Preston Omondi
- Kim McCarthy

Thank You

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