Prototype RT-qPCR PML::RARA Reagents and a Commercial PML-RARA Kit Yield Highly Correlated Results

H063

Walairat Laosinchai Wolf, Adrian Lara, and Justin T Brown

Asuragen, a Bio-Techne brand, Austin, TX

Summary

- We developed a PML::RARA prototype assay to detect short (bcr3), long (bcr1), and some variable (bcr2) forms in a single reaction.
- The prototype is a multiplex RT-qPCR performed in singleton with less than five hours from sample-to-answer, reducing reactions per sample compared to other methods.
- Asuragen's prototype assay and the *ipsogen* PML-RARA bcr1 kit (Qiagen) were highly correlated.
- The lack of inter-laboratory standards limits the interpretation of assay-specific quantification differences.

Introduction

Quantification of PML::RARA fusion transcripts of translocation t(15;17) assesses tumor burden in studies for molecular remission in acute promyelocytic leukemia (APL). Measurement differences between methods might influence scientific interpretations. Hence, characterizing variation between methods is an important area of study. Here, we developed a prototype assay and compared performance to a commercial kit, demonstrating correlation and bias between two assays. The prototype assay demonstrated performance on par with a commercial kit while also achieving significant improvements in workflow and reactions per sample.

Materials and Methods

We developed a prototype RT-qPCR assay (Asuragen). Calibrators and controls were composed of *in vitro* transcripts for PML::RARA short (bcr3) and long (bcr1) forms, respectively, multiplexed with *in vitro* transcripts for the endogenous normalizer ABL1. Both RT and qPCR were performed on the ABI 7500 Fast Dx instrument. The *ipsogen* PML-RARA bcr1 Kit (Qiagen, RUO) was used according to its accompanying protocol[1] on the ABI 7500 Fast Dx instrument. The Qiagen kit is performed in simplex (PML::RARA and ABL1 in separate reaction wells) and in duplicate per sample. It requires 4 reactions per sample measurement. To compare methods, we formulated a non-blinded challenge panel of NB4 cell line RNA (bcr1 long form) blended with non-leukemic human donor RNA targeting a range of percent ratio from 10 to 0.001 (n=23 possible measurements per method).

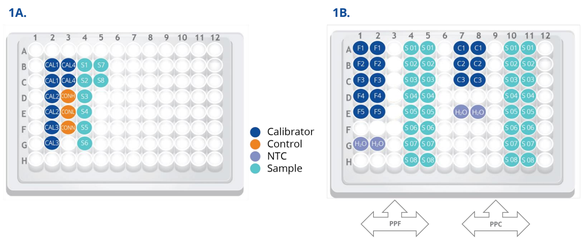

Figure 1. qPCR Plate Setup for Each Assay Across Eight Samples. A. Asuragen Prototype. Multiplexed design amplifies and detects PML::RARA fusion and a control gene in the same reaction. **B. Qiagen Kit.** Simplex design amplifies and detects PML::RARA and a control gene in separate reaction wells. Testing requires duplicates per reaction.

Table 1. Analytical Values for % Ratio and LR Scales. The Log Reduction (LR) values used in the studies herein are the log10 reduction from 100%. Therefore, LR = 2 – log10(%ratio). The table provides a summary of several LR values and their corresponding %ratio values for reference. We note that the international scale values of %IS are well established for BCR::ABL (major breakpoints) but has not been defined for PML::RARA.

%PML::RARA:ABL1	Log reduction (LR)
100	0.0
10	1.0
1	2.0
0.1	3.0
0.01	4.0
0.0032	4.5
0.001	5.0

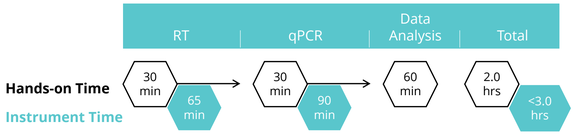

Figure 2. Assay Workflow. An Asuragen prototype assay requires 2 hours hands-on time and <3 hours instrument time excluding RNA extraction.

Table 2. Workflow Comparison of Asuragen Prototype and Qiagen Kit. Asuragen prototype is designed for a simple RT and qPCR setup and all components are included in the kit. Asuragen prototype utilizes IVT RNA for standard curve to account for RT efficiency. Controls (IVT RNA) are provided in the Asuragen prototype.

	Asuragen prototype	Qiagen kit	Extra pipetting steps with Qiagen kit
Pipetting steps for RT	2 for RT master mix 3 including RNA transfer per sample	7 for RT master mix 11 including RNA transfer per sample	5 for RT master mix 8 including RNA transfer per sample
Pipetting steps for qPCR	3 for qPCR master mix 4 including cDNA transfer per sample	3 for qPCR master mix 7 including cDNA transfer per sample	3 including cDNA transfer per sample
Standard curve	IVT RNA	Plasmid DNA	2 for PML::RARA 6 for ABL1

Results

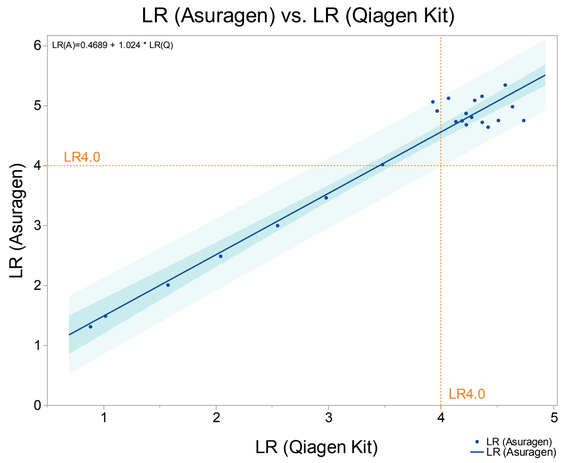

Figure 3. Correlation Plot comparing Asuragen Prototype and Qiagen Kit. This analysis includes all log reduction (LR) values (LR=2-log10(%ratio)). Slope of the linear regression line is 1.024 and Pearson R correlation coefficient is 0.977 (95%CI: 0.945, 0.990), p<0.0001. Confidence of fit is shown in darker color, with 95% limits of agreement (LOA) shown in light blue. Dotted lines represent LR4.0 or 0.01 %ratio.

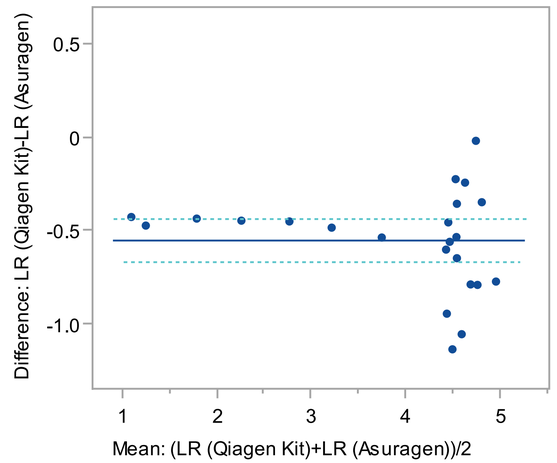

Figure 4. Bias Plot of Asuragen Prototype and Qiagen Kit. This analysis includes all log reduction (LR) values. X-axis represents the mean of both test's LR values and Y-axis represents the difference as LR(Qiagen Kit) – LR(Asuragen). The solid blue line is drawn at the mean difference with 95% confidence shown in dotted blue lines. Dotted orange lines show the upper limit of agreement (LOA) of -0.031 (-0.2315, 0.1690) and the lower LOA of -1.078 (-1.2779, -0.8775), with 21/23 (91.3%) within these limits. Bias appeared potentially non-uniform by visual inspection. Asuragen test's LR values were on average 0.555 higher than Qiagen test's LR values.

Table 3. Contingency Analysis of Qualitative Detection. All valid test results are included. Ten duplicate-discordant PML::RARA results (one positive, one negative) generated by the Qiagen test are included. After doing so, all test samples were deemed positive in both assays (no Undetected results). Percent agreement across both assays was 86.96% (20/23). Three samples near the LR4.0 cutoff were below LR4.0 for the Qiagen test while above LR4.0 for the Asuragen test. Cohen's Kappa Coefficient = 0.709 (95%CI: 0.415, 1).

	Qiagen LR		
Asuragen LR	≤4	>4	**Total**
≤4	6	0	6
>4	3	14	17
Total	9	14	23

Conclusions

- Positive values yielded a linear regression with slope of 1.024 and a Pearson R correlation coefficient of 0.977.
- Since no international harmonization efforts for standardized measurement have occurred for PML::RARA, the mean bias of about 0.6 logs (3.6 fold) is of unknown significance.
- Percent agreement of detection across both studies was 86.96% with ten duplicate-discordant PML::RARA results (one positive, one negative) generated by the Qiagen test.
- The Asuragen PML::RARA prototype demonstrates comparable performance to the Qiagen kit with four times fewer reactions per sample and a simplified workflow.

Reference:

1. ipsogen PML-RARA bcr1 Kit Handbook.

Presented at AMP 2023

Scan code to learn more

bio-techne®

Asuragen®