# Standardized Nucleic Acid qPCR BCR-ABL Pilot Study

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Purpose: CML treatment monitoring using PCR based peripheral blood testing provides improved test sensitivity over cytology but suffers from inadequate standardization in most labs due to variations inherent in the existing PCR methodologies. This report presents the initial analytic performance evaluation of a novel competitive template based peripheral blood b2a2/b3a2 transcript abundance method. Standardized Nucleic Acid Quantification BCR-ABL (SNAQ-BCRABL) uses mixtures of b2a2 or b3a2 and GusB competitive templates and melting curve analysis to provide clinically needed quality controls to correct for operator, instrument, sample, and reagent variation when measuring transcript abundance. The SNAQ-BCRABL pilot study is a 50 CML patient study that looks at the imprecision and linearity of two established CLIA real-time qPCR assays and SNAQ-BCRABL.

## NEED FOR MOLECULAR CML TESTING STANDARDIZATION

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Currently, the quantitative definition of a significant "rise" in *BCR-ABL* RNA (to warrant mutation analysis) is controversial and varies among laboratories.

However, because PCR negativity is *poorly standardized among laboratories* and varies depending on the sensitivity of PCR assays, the definition of CMR continues to evolve, and confirming a consistent prognostic value for CMR has been problematic.

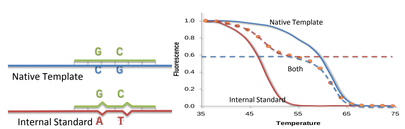
blood

2010 116: e111-e117

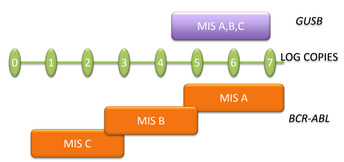
RE: International Standardization….

While this process has generally worked well, it is apparent that the establishment of conversion factors is time-consuming, complex, expensive, and open to only a limited number of laboratories at any given time. Furthermore, it is unclear how frequently any individual CF will need to be revalidated.

## Standardized Nucleic Acid qPCR (SNAQ)

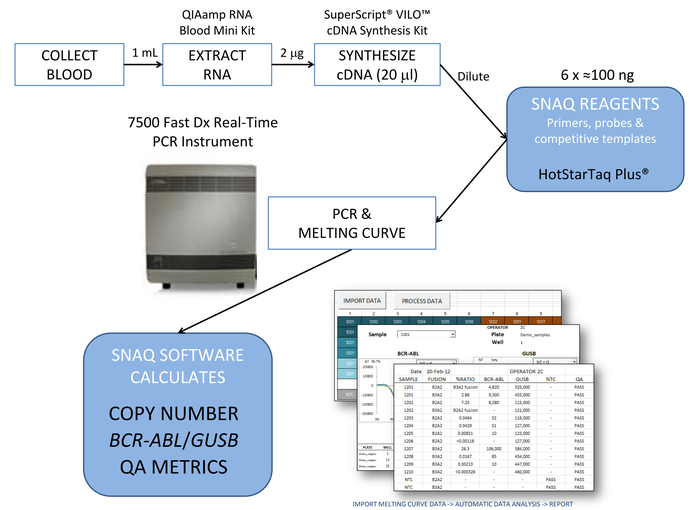



SNAQ Quantification by Ratio Analysis. The cDNA sample contains native template (blue) which is spiked with a known quantity of internal standard (red) to which a fluorescent probe (green) may hybridize. The two templates are kinetically identical by PCR, so their ratio is maintained during amplification and melting curve analysis. During melting curve analysis, the probe fluorescence decreases as it dissociates from template, the Tm difference between internal standard and native template arises from the base pair mismatch between internal standard and probe. Intermediate melting profiles (orange circles) indicate presence of both templates, the contribution of each derived by curve fitting (red dashed line, internal standard; blue dashed line, native template). Native transcript abundance is calculated from the known internal standard quantity spiked into the PCR and the resulting ratio.




A single internal standard supports a dynamic range of about 1000-fold. SNAQ BCR-ABL MBr achieves seven logs dynamic range by mixing specimen with three different Mixtures of Internal Standards (MIS). MIS A, B & C each have *GUSB* internal standard at $4x10^5$ copies (purple box), and *BCR-ABL* internal standards at $8x10^5$, $6x10^3$ & 50 copies (orange boxes, the dynamic range of each MIS is indicated on the above plot). *GUSB* transcript abundance measurements in every PCR provides a QA to ensure sufficient sample loading. Using a mixture of reference and target gene(s) in a competitive PCR ensures that the *BCR-ABL/GUSB* results are consistent despite PCR efficiency and volumetric variation.

## SNAQ BCR-ABL WORKFLOW




Blood is collected and shipped to laboratory for RNA extraction and cDNA synthesis. cDNA is divided into three mixtures of internal standard (MIS A, B & C), *GUSB* & *BCR-ABL* primer/probes & mastermix (100 ng/ PCR). Samples are amplified and melting curve data collected and exported to a spreadsheet where melt curves are fit to a hybridization model to generate a native template : internal standard ratio and QA metrics (sample load, product specificity). This ratio is used to calculate transcript abundance, from which *BCR-ABL/GUSB* ratio is calculated.

## STUDY DESIGN

**Eligibility of Subjects**

*Subject characteristics*: (data will be collected for each sample)
**Age:** >18
**Ethnicity:** Any (Reflect the geo-ethnicity makeup of the United States.**)**
**Gender:** M or F
**Other characteristics**
Know identity of either b2a2 or b3a2 CML fusion

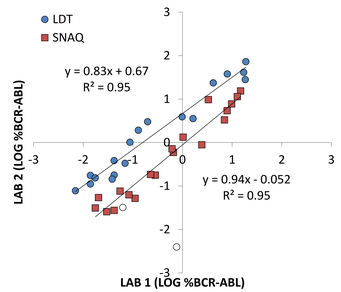
**Inclusion Criteria:**
CML patients expected to have PCR positive b2a2 and/or b3a2 results. Willing to provide 2x5mL blood drawn on or within a day that MD Anderson collected samples for their real-time qPCR CML testing.
Participants' willingness to give and sign informed consent.
**No exclusion criteria.**
**Length of subject's participation in the study may include a single visit for a blood draw. Patients who agree may have repeat samples drawn in subsequent visits planned for their routine clinical care.**

**Research Plan & Methods**
Up to fifty samples may be collected for this study. The patient recruitment and screening will be performed by the doctors participating in the study. Remove two 5 ml EDTA tubes of blood from arm vein. This blood volume will be collected once and assigned a unique de identified study number that will allow patient association with MD Anderson BCR-ABL RT-qPCR report. Blood samples will be stored and shipped at 4C. Shipments will include a requisition sheet and an email with a scanned completed requisition form to accugenbcrablnotification@cancergenetics.com. Both tubes will have their BCR-ABL levels quantified using the SNAQ method; one tube will be processed at MD Anderson Cancer Center under the supervision of Dr. Raja Luthra, the other tube will be received by Cancer Genetics, Inc. within the first 24 hours of blood draw. All samples must be processed up to the WBC lysis step within 48 hours of blood draw as indicated in the SNAQ BCR-ABL product insert. WBC lysates may be stored at <-70C in support of batch processing as indicated in the QIAGEN QIAamp RNA Blood Mini Kit protocol. The SNAQ method involves RNA isolation, conversion to cDNA, PCR amplification, melting curve analysis of BCR-ABL and GUSB products, and report generation using AccuGenomics SNAQ software. MD Anderson will provide information of which alleles (e.g., b2a2 and/or b3a2) are identified for each specimen. MD Anderson will send electronic copies of the RT-qPCR and SNAQ reports to AccuGenomics for data analysis.

## INTERLABORATORY CORRELATION

| | LDT | | SNAQ | |
|---|---|---|---|---|
| CATEGORY | LAB1 | LAB2 | LAB1 | LAB2 |
| NEGATIVE | 11 | 10 | 8 | 7 |
| LOW POSITIVE | 4 | 0 | 1 | 1 |
| QUANTITATIVE | 20 | 26 | 20 | 21 |
| HIGH POSITIVE | 1 | 0 | 0 | 0 |
| KENDALL TAU-B | 0.83 | | 0.91 | |

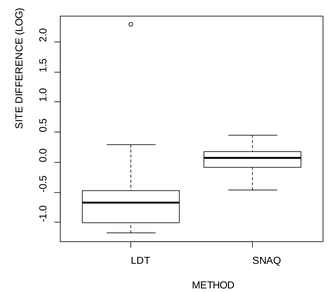
**Purpose:** Compare the test results for paired LDT or paired SNAQ samples.
**Method:** Kendall Tau-b is a ranked correlation that allows ties, thereby enabling the incorporation of qualitative results into the analysis (negatives =0, low positives = 0.001, High Positives = 1000).
**Results:** The LDT method had a lower tau-b correlation than the SNAQ method (0.83 vs. 0.91). The lower LDT correlation appears to have arisen from a lower analytical sensitivity of one LDT method.
**Conclusion:** SNAQ test had a better ranked correlation than the LDT method.




**Purpose:** Characterize the LDT and SNAQ interlaboratory correlation for paired quantitative samples (samples in the reporting range of each method).

**Results:** To provide a better estimate of the interlaboratory LDT response , two LDT outliers (open circles) were removed from the linear analysis. The LDT correlation log vs. log plot (filled circles) had an intercept that indicated a significant bias of 0.67+/- 0.059 (n=18). Further, the LDT slope of 0.83+/-0.046 indicated the bias changes as a function of [BCR-ABL]. The SNAQ method had a near zero intercept (0.052 +/- 0.057, filled squares) and near 1:1 response of 0.95 +/-0.055 (n=18). Further samples will be required to determine how close the SNAQ slope is to the ideal 1.0.
**Conclusion:** The SNAQ method had a more desirable interlaboratory bias (<12%) than the LDT method (>4-fold). These results indicate the implementation of International Standard reporting as a method to control for interlaboratory variation was not working as desired.

## ANALYTICAL ACCURACY ESTIMATE




**Purpose:** Estimate the analytical accuracy of the LDT and SNAQ methods.
**Method**: For paired samples with quantitative results, calculate the average and standard deviation of the fold difference for each method. This approach combines the systematic and response bias into a single accuracy estimate. Accuracy = average diff + 2 x SD diff
**Results:** For visual comparison, the plot is a boxplot of the interlaboratory log difference paired by method. Two outliers for the LDT method were removed to provide a better estimate of analytical accuracy. The average fold difference for LDT was 7.1 +/-4.4 fold (n=18), which corresponds to an accuracy of 16-fold. The SNAQ method had and average of 1.24 +/-0.62 (n=18) fold, which corresponds to a 2.5-fold accuracy.
**Conclusion:** Despite the removal of two outliers, the combined bias and imprecision of the LDT methods would support a 16-fold accuracy. The SNAQ method demonstrated 2.4-fold accuracy.

## SUMMARY

Reporting in International Standard units was intended to standardize CML treatment monitoring by controlling for bias between molecular testing laboratories. In this pilot study, the International Scale results from two CLIA laboratories had a 95% confidence of 16-fold. An alternative BCR-ABL monitoring method, SNAQ-BCRABL, was designed to control for the analytical variation arising from reagents, users, sample quality and instrument, and produced more consistent interlaboratory results than the LDT methods (2.4-fold accuracy). Further, the LDT produced two unexplained outlier results whereas the SNAQ method had no outliers.
In future, additional patients will be recruited to allow for better estimates of SNAQ's imprecision. In addition, this study revealed that the MIS A reagent was not required for accurate BCR-ABL quantification, therefore its replacement with one additional MIS B & C replicate is expected to further improve SNAQ accuracy.