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# Standardizing *BCR-ABL* MBr Monitoring Without External Standards

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**Abstract**: *BCR-ABL* blood transcript abundance is a key marker for monitoring tyrosine kinase inhibitor treatment of chronic myelogenous leukemia (CML). Standardization of *BCR-ABL* qPCR measurements suffer from variability introduced by sample purity, preparation, reagents and instrument. International standardization efforts have made modest improvements but rely on cumbersome external reference material and lab calibration. This poster describes initial testing of a novel qPCR method that controls for the majority of real-time qPCR variation.

## NEED FOR MOLECULAR CML TESTING STANDARDIZATION

The Oncologist Leukemias

***The Oncologist*** 2010;15:744–74

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.

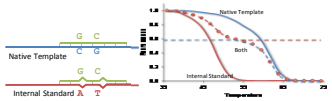
blood

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.

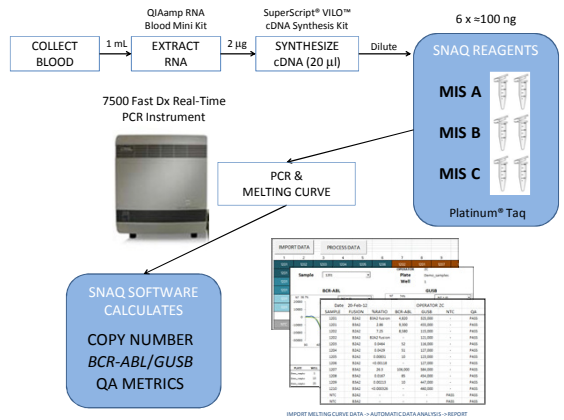
## Standardized Nucleic Acid qPCR (SNAQ)




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.

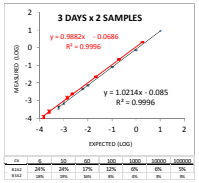

A single internal standard supports a dynamic range of about 1000-fold. SNAQ BCR-ABL MBr achieves seven logs dynamic range by mixing specimen with three different Mixtures of Internal Standards (MIS). MIS A, B & C each have *GUSB* internal standard at 4x10$^5$ copies (purple box), and *BCR-ABL* internal 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 MBr WORKFLOW

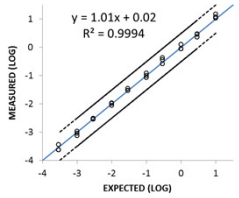



Blood is collected and shipped to laboratory for RNA extraction and cDNA synthesis. cDNA is divided into three mixtures of internal standard (MIS A, B & C), *GUSB* & *BCR-ABL* primer/probes & mastermix (100 ng/PCR). Samples are amplified and melting curve data collected and exported to a spreadsheet where melt curves are fit to a hybridization model to generates 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.

## cDNA LINEARITY & IMPRECISION




**Purpose**: Observe SNAQ BCR-ABL MBr cDNA quantification linearity and interday reproducibility.
**Method**: Cell line cDNA mixtures derived from KCL22 (a b2a2 source, black line) K562 (b3a2, red line) and HL60 (*BCR-ABL* negative) were quantified for *BCR-ABL* and *GUSB* using a CE based BCR-ABL StaRT-PCR. Serial dilutions generated stocks of 10$^5$, 10$^4$, 10$^3$, 10$^2$, 60, 10 and 6 *BCR-ABL* copies and 10$^4$ *GUSB*. On each of three days two aliquots were measured by SNAQ BCR-ABL MBr method (y-axis, std error bars) and plotted against expected answer (x-axis). Tables indicates interday replicate CV.
**Results**: The measured vs. expected responses for both reagents demonstrated a slope of 1.0 ($R^2$ = 1.00) with a near zero intercept, indicating a near perfect correlation between measured and expected answer. The interday replicate CV ranged from 3 to 24%, with the CV rising below 100 *BCR-ABL* starting copies.
**Conclusion**: Both p210 transcripts were accurately measured over a 4-log reduction of *BCR-ABL* with interday variation <25%.




**Purpose:** Determine if three laboratories could measure cDNA reference samples within a half a log of expected answer.
**Method**: Serially diluted cDNA samples were distributed frozen to three laboratories for SNAQ BCR-ABL MBr measurement (circles). Two sites used ABI 7500 Fast Dx and one used standard ABI 7500. %*BCR-ABL/GUSB* ratio measurement (y-axis) must be within a half a log (solid diagonal lines of plot) of expected answer (x-axis, blue line) based on dilution.
**Results**: All ten sample measurements fell within +/- 0.17 logs of expected, well within the +/-0.5-log requirement for the method. The median interlaboratory CV for each sample was 15% (2-22% range).
**Conclusion**: The results were consistent with measuring *BCR-ABL* over five logs dynamic range with better than half-log accuracy.

## CLINICAL SPECIMEN MEASUREMENTS

| SAMPLE | SITE 1 | SITE 2 | SITE 3 | MEAN | STD | CV |
|---|---|---|---|---|---|---|
| RNA1 | 22.83 | 26.90 | 25.90 | 25.21 | 2.13 | 8% |
| RNA2 | 7.46 | 6.78 | 7.83 | 7.35 | 0.53 | 7% |
| RNA3 | 0.14 | 0.20 | 0.16 | 0.17 | 0.03 | 18% |
| RNA4 | 55.57 | 75.44 | 62.02 | 63.82 | 10.14 | 16% |
| RNA5 | 0.10 | 0.15 | 0.14 | 0.13 | 0.03 | 22% |
| RNA6 | 6.30 | 10.46 | 9.07 | 8.42 | 2.12 | 25% |

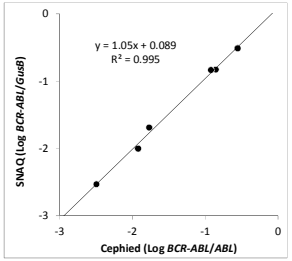
**Purpose**: Examine interlaboratory imprecision of six clinical RNA specimen.

The goal of this experiment was to demonstrate that interlaboratory imprecision would support claims of half-log accuracy (i.e., CV <150%) when measuring archived CML patient samples.

**Method**: RNA aliquots from three b2a2 and three b3a2 archived CML patient samples, representing high, medium and low *BCR-ABL* as characterized by a CLIA LDT testing, were distributed to three laboratories. Each lab used the same protocol for cDNA synthesize and SNAQ BCR-ABL MBr measurement. Table indicates the %*BCR-ABL/GUSB* measurements from each site, mean, standard deviation and %CV.

**Results**: The interlaboratory CV ranges for all six samples spanned 8% to 25%, better than the 150% CV requirements necessary for half-log accuracy.

## METHOD CONCORDANCE




**Purpose:** Compare SNAQ BCR-ABL MBr and Cepheid method concordance using clinical specimen.
**Method**: p210 and ABL transcripts abundance of twenty-three CML patient whole blood samples were measured using the *BCR-ABL* assay in the Cepheid GeneXpert® system (x-axis). Frozen cell pellets were made from the remaining blood specimen. RNA was isolated from the frozen cell pellets and provided blinded for p210 fusion (b2a2 or b3a2) identification and *BCR-ABL/GUSB* transcript abundance measurements using the SNAQ BCR-ABL MBr method. Concordance analysis was performed on unblinded data.

**Results**: *GUSB* transcript abundance measurements indicated poor RNA yield (median 7747 *GUSB* copies/PCR, range 649-17697 copies). Six of 23 samples gave detectable *BCR-ABL* (y-axis) with a significant inter platform concordance ($R^2$=0.995) over a 2-log sample range. Analysis of unblinded Cepheid measurements indicated that the 17 samples with no detectable *BCR-ABL* by the SNAQ BCR-ABL MBr method would contain less than 3 copies *BCR-ABL* per PCR (i.e., below PCR LOD). SNAQ b2a2 and b3a2 identification of the six *BCR-ABL* positive specimen matched clinical records.
**Conclusion**: SNAQ method transcript abundance and fusion identification correlation with an established clinical methods was excellent. The benefit of absolute *GUSB* quantification was demonstrated in the identification of specimen with insufficient template to ensure accurate characterization of low [*BCR-ABL*].

## SUMMARY

SNAQ BCR-ABL MBr reagent beta test demonstrated an excellent interday, interlaboratory reproducibility sufficient for 2-fold interlaboratory accuracy claims without reliance on external reference material. Follow up studies will gauge impact of sample collection & RNA isolation. Further, the SNAQ method should be applicable for other qPCR applications.