# Modifications to RNA Isolation Protocols Meet Requirements for Modern CML Monitoring of BCR-ABL1 Transcript Levels

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## Summary

- A 4.5-log reduction of BCR-ABL1 transcript levels (MR4.5 or 0.0032% on the International Scale) is associated with deep molecular response and potential treatment-free response of Ph+ CML.
- This level of analytical sensitivity requires a sufficient quantity and concentration of RNA in the test reaction for reverse transcription and quantitative PCR analysis (RT-qPCR). Standard protocols for isolating RNA from whole blood, however, may yield RNA that is too dilute to achieve such analytical sensitivity.
- The purpose of this study was to increase RNA yield and concentration from commercially available RNA isolation kits as a model approach for use in routine CML monitoring.
- Modifications to commercially available isolation kits improved RNA yield and concentration and were easily implemented to achieve reliable and consistent RNA concentration of at least 100 ng/µL.

## Introduction

Although qPCR-based techniques can meet the sensitivity requirements to properly monitor CML patients at ≥MR4.5, there are pre-analytical best practices that must be considered in order to ensure that the required amount of total RNA from isolated white blood cells will facilitate low-level detection of BCR-ABL1 transcripts. Unless sufficient sample volumes are collected for RNA extraction and put into the qPCR assay, expected sensitivity may not be achieved. Global guidelines established for harmonization of molecular monitoring of CML recommend standardization of key critical steps in sample preparation. The QuantideX® qPCR BCR-ABL IS Kit recommends that purified total RNA be evaluated for concentration (OD260 indicating a concentration of ≥100 ng/µL) and purity (as estimated by OD260/OD280 ratio >1.6 and OD260/OD230 ratio >1.2) by standard spectrophotometric methods.

## Methods

To target an RNA concentration of at least 100 ng/µL and purity estimated by OD260/OD280 ratio >1.6 and OD260/OD230 ratio >1.2, factors such as input blood volume, addition of Proteinase K, elution volume, and elution approach were evaluated on three commercially available RNA isolation kits.

QIAamp RNA Blood Mini Kit (QIAGEN, P/N 52304) was used for primary isolation experiments. Kit guidelines specify that for up to 1.5 mL of blood input (generally up to 10 million white blood cell input), to use elution volumes from 30 to 100 µL to achieve 1 to 5 µg yield. The manufacturer-recommended protocols and specific modifications were evaluated. 1.5 mL and 3.0 mL of whole blood specimens were compared. Additionally, we tested 30 µl, 50 µl, 80 µl, and 100 µl elution volumes with 1 minute and 5 minute incubation periods. Double elution techniques were assessed by passing the eluate over the filter for a 2nd elution with comparison to single elution and two separate elution methods. Other test conditions included eliminating the shredder column and adding an additional RPE wash step. The addition of Proteinase K (PK) lysis step was tested. PK was added to white blood cell lysate and samples were incubated at 55˚C for 15 minutes with 1500 rpm shaking. Additional PK testing was done on 1.5 mL and 3 mL blood input volumes.

The Leukocyte RNA Purification Plus Kit (Norgen Biotek Corp., P/N 21250 ) was evaluated as a secondary manual isolation solution, using both the manufacturer-recommended protocol and a modified protocol. 2.5 mL of whole blood was used from 24 donors. After the initial eight donors tested failed to produce RNA with sufficient concentration, modifications were made to the procedure and 16 additional donors were tested. Red blood cell lysis incubation was adjusted from 5 to 10 minutes. White cell pellet centrifugation speed and time was increased from 200 x g for 3 minutes to 500 x g for 10 minutes for the modified procedure. Two elution steps were performed for each sample in separate microcentrifuge tubes (1st elution at 30 µL and 2nd elution at 20 µL). Eight donors were tested in duplicate using the kit protocol for a total of 16 data points. Two batches of 8 additional donors were tested in duplicate with the modified protocol for a total of 32 data points.

A Maxwell® RSC Instrument (Promega, P/N AS4500) was used with the Maxwell® RSC simplyRNA Blood Kit (Promega, P/N AS1380) to evaluate a semi-automated isolation method using 2.5 mL whole blood specimens from 16 donors. A total of 30 total data points were collected.

| Manufacturer | Manufacturer's Recommendations | Modifications/Test Conditions | Rationale | Observations |
|---|---|---|---|---|
| Qiagen | Max 1.5 mL whole blood input | 1.5 mL vs 3 mL whole blood input | Increasing the whole blood input should increase RNA yield | Higher input increased RNA yield |
| | 30-100 µL elution volume | 30, 50, 80, 100 µL elution | Optimizing elution volume to meet target concentration | 50 µL elution increased yield with minimal negative effect on concentration |
| | No incubation of eluate specified | 1 vs 5 min of eluate incubation | Maximize RNA recovery from column | No effect |
| | Single elution | Single elution vs 2 separate elutions vs double pass of same elute | Maximize RNA recovery from column | Repass increased RNA yield |
| | Use column shredder | Test no shredder | Minimize RNA loss in up-front processing | No effect |
| | RPE wash 2X | 3X RPE wash | Improve 260/280 and 260/230 purity ratios | No effect |
| | No proteinase K | Proteinase K treatment | Improve leukocyte lysis | PK treatment increased RNA yield |
| Norgen | 3-5 min. RBC lysis incubation | 10 min. incubation | Longer lysis to ensure RBC lysis | Simultaneously tested modifications increased RNA yield |
| | Leukocyte pellet centrifugation 200xg for 3 min. | 500xg for 10 min. | Maximize WBC pellet formation | |
| | Centrifuge after DNase treatment | No centrifugation after DNase treatment | To minimize column drying | |
| | 10-20 µL elution | 20 µL elution | Maximize RNA recovery from column | |
| | Recommended 2nd elution - 10-20 µL | 30 µL 2nd elution | Maximize RNA recovery from column | |
| Promega | No modifications from manufacturer's recommendations | | | |

**Table 1. Methods and Modifications.** Three commercially available kits were tested with the ultimate goal of identifying a procedure for Blood RNA isolation that achieves >100 ng/µL in at least 30 µL for all samples and purity estimated by OD260/OD280 ratio >1.6 and OD260/OD230 ratio >1.2. In comparing these kits, RNA produced must meet both the concentration and purity metrics to be considered passing.

### Observations

1. QIAamp RNA Blood Mini Kit (QIAGEN, P/N 52304) was used for primary isolation experiments. Multiple conditions were tested with a goal of increasing RNA concentration to >100 ng/µL . Five subsequent experiments were designed and completed across 181 separate RNA isolations. Increasing whole blood input volume to 3 mL, using 50 µL volume for elution and passing elute over the column for a second time, and treating white cell pellet with proteinase K produced the most consistent increase in RNA yield and concentration. The effect of PK on purity was unclear.
2. Leukocyte RNA Purification Plus Kit (Norgen Biotek Corp., P/N 21250) was evaluated as a secondary manual isolation solution, using both the manufacturer-recommended protocol and a modified protocol. Three experiments were completed across 48 separate RNA isolations. 2.5 mL of whole blood was used for each isolation from 24 donors. After the initial eight donors (14 out of 16 RNA isolations) failed to produce RNA with sufficient concentration, modifications were made to the procedure and 16 additional donors were tested. 2 batches of 8 additional donors were tested in duplicate with the modified protocol for 32 separate RNA isolations; 32/32 yielded RNA concentration >100 ng/µL.
3. Maxwell® RSC Instrument (Promega, P/N AS4500) was used with the Maxwell® RSC simplyRNA Blood Kit (Promega, P/N AS1380) to evaluate a semi-automated isolation method using 2.5 mL whole blood specimens from 16 donors, 30 total data points were collected; 27/30 yielded RNA concentration >100 ng/µL.

## Results

| Test Conditions | Average Concentration (ng/µL) |
|---|---|
| PK treatment with double pass elution | 206 |
| No PK treatment with double pass elution | 152 |
| PK treatment with single pass elution | 101 |

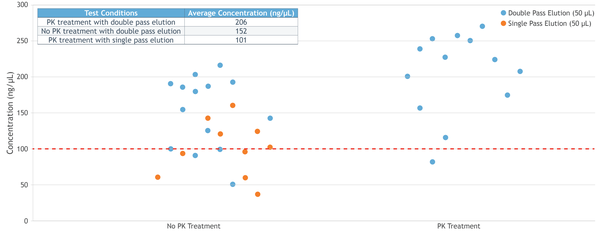
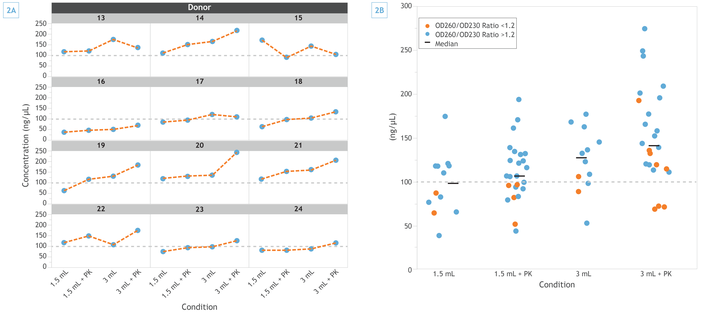



**Figure 1. QIAamp RNA Blood Mini Kit (QIAGEN, P/N 52304) was used on 37 Replicate Lysate Samples from Pooled Whole Blood Across Two Experiments.** For 27/37 replicates a double pass elution strategy was employed; 24/27 replicates yielded RNA >100 ng/µL. 13/37 replicates were treated with Proteinase K (PK) lysis step and followed the double pass elution strategy. PK was added to white blood cell lysate, incubated at 55C for 15 minutes with 1500rpm shaking. 12/13 PK treated replicates yielded RNA >100 ng/µL.

### Observations

1. The top 20% of replicate sample with the highest RNA concentration were treated with PK and followed the double pass elution strategy
2. The average RNA concentration of replicates treated with PK and that followed the double pass elution strategy was 204% higher than replicates without PK treatment that followed the double pass elution strategy and 151% higher than replicates that were not PK treated but did follow the double elution strategy.




**Figures 2A and 2B. QIAamp RNA Blood Mini Kit (QIAGEN, P/N 52304) was used to Isolate RNA from Whole Blood Collected from 12 Donors, 6 Isolations Each, Split Across 4 Batches.** For each donor, 3 replicate isolations with 1.5 mL blood input and 3 replicate isolations with 3 mL blood input were performed. For each volume condition, 1 replicate was run without PK digestion and 2 replicates included PK. Double pass of 50 µL elute was used for all replicates.

### Observations

1. For 9 of 12 donors, the highest RNA concentration in the elution came from the 3 mL blood input + PK (average of 2 reps).
2. For 10 of 12 donors, 3 mL + PK yielded RNA >100 ng/µL in 2/2 replicates, and for 1 donor in 1/2 replicates.
3. 16/72 RNA samples isolated failed to meet OD260/OD230 ratio >1.2; of these 16, 12 were treated with PK.
4. For 9 of the 12 donors using 3 mL of blood input without PK yielded RNA >100 ng/µL.

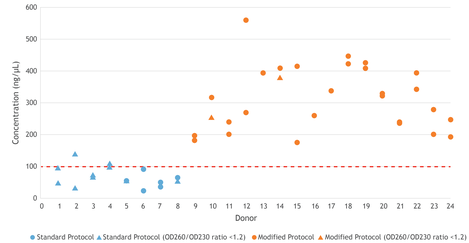



**Figure 3. Leukocyte RNA Purification Plus Kit (Norgen Biotek Corp., P/N 21250) was used to Isolate RNA from 2.5 mL of Whole Blood Collected from 24 donors, Across 3 Experiments.** Donors were tested in duplicate. After the standard protocol failed to produce RNA with sufficient concentration, modifications were made to the procedure and 16 additional donors were tested. 2 batches of 8 additional donors were tested in duplicate with the modified protocol for 32 separate RNA isolations; 32/32 yielded RNA concentration to >100 ng/µL. A single pass of 30 µL of elution buffer was used.

### Observations

1. 2/16 RNA samples isolated with the standard protocol yielded RNA >100 ng/µL.
2. 10/16 RNA samples isolated with the standard protocol failed to meet OD260/OD230 ratio >1.2; 100% of the samples met OD260/OD280 ratio >1.6
3. 100% (32/32) of the RNA samples isolated with the modified protocol yielded RNA >100 ng/µL; 28/32 yielded RNA >200 ng/µL.
4. 2/32 RNA samples isolated with the modified protocol failed to meet OD260/OD230 ratio >1.2; 100% of the samples met OD260/OD280 ratio >1.6.
5. When the modified protocol was used, the sample pass rate increased from 0% to 93.75%. Pass metrics are defined as >100 ng/µL, OD260/OD280 ratio >1.6, and OD260/OD230 >1.2.

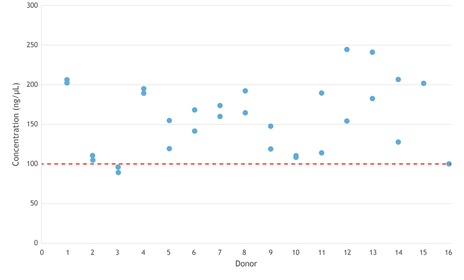



**Figure 4. Maxwell RSC Instrument (Promega, P/N AS4500) was used with the Maxwell RSC simplyRNA Blood Kit (Promega, P/N AS1380) to Evaluate a Semi-Automated Isolation Method using 2.5 mL Whole Blood Specimens from 16 Donors, 30 Total Data Points were Collected; 27/30 yielded RNA Concentration to >100 ng/µL.** Two batches of 8 donors were tested. Donors were tested in duplicate with the exception of donors 15 and 16 due to limited kit resources. A single elution of 50 µL was performed by the instrument.

### Observations

1. Using the manufacturer's recommended input of 2.5 mL whole blood on the semi-automated RSC instrument resulted in 90% of samples (27/30) meeting the minimum input requirement of 100 ng/µL with acceptable quality ratios.

## Conclusions

- Modifications to commercially available isolation kits improved RNA yield and concentration and were easily implemented to achieve reliable and consistent RNA concentration of at least 100 ng/µL and purity estimated by OD260/OD280 ratio >1.6 and OD260/OD230 ratio >1.2.
- The factors of blood volume, pre-treatment, and elution method were extensible to both magnetic bead-based, automated extraction methodologies, and column-based manual methodologies.
- These modifications and improved RNA yield address critical pre-analytical factors for routine CML monitoring that support accurate assessment of deep molecular response and eligibility for treatment cessation.

Conflict of Interest Disclosure. All authors have the financial relationship to disclose: Employment by Asuragen. QuantideX® qPCR BCR-ABL IS Kit (item 86003) is CE-marked IVD. QuantideX® qPCR BCR-ABL IS Kit (item 49574) is FDA-cleared IVD.
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