

Detection of BCR-ABL1 Kinase Domain Mutations Causing Imatinib Resistance in Chronic Myelogenous Leukemia

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Abstract

The reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34;q11), Philadelphia chromosome] creates a BCR-ABL1 fusion protein that occurs in approximately 95% of cases of chronic myelogenous leukemia (CML), 15% of cases of adult acute lymphoblastic leukemia, and 5% of adult cases of acute myeloid leukemia. The BCR-ABL1 protein is a constitutively activated tyrosine kinase that induces and maintains the neoplastic phenotype in these leukemias. PCR-based methods to identify and quantitate the tumor-specific BCR-ABL1 RNA have been shown to be an ultrasensitive diagnostic, prognostic, and monitoring tool for Philadelphia-positive leukemias. A novel tyrosine kinase inhibitor (TKI), imatinib, has been confirmed as an effective targeted treatment in most CML patients. However, a significant minority of patients being treated with imatinib develop resistance to the drug as evidenced by rising BCR-ABL1 levels. The most common mechanism of resistance in these patients is the development of mutations in the BCR-ABL1 kinase domain (KD) that abrogate binding of imatinib. Although KD mutations are quite heterogeneous, the identification of the exact mutation site is clinically important, as some mutations, but not others, can be effectively treated with second-generation TKIs. One mutation, T315I, for example, renders the leukemia resistant to all first- and second-line TKIs. Thus, DNA sequencing of the BCR-ABL1 kinase domain in resistant patients helps identify those who may benefit from a change in TKI agents, or those who should be considered for other therapeutic measures, such as stem cell transplantation. We describe here a method for sequencing the BCR-ABL1 kinase domain in peripheral blood or bone marrow of CML patients.

Key words Chronic myelogenous leukemia, Philadelphia chromosome, BCR-ABL1, Imatinib, Dasatinib, Nilotinib, Tyrosine kinase inhibitors, Major molecular response

1 Introduction

Chronic myelogenous leukemia (CML) is characterized by a reciprocal chromosome translocation between chromosomes 9 and 22. This translocation creates a fusion protein consisting of BCR and the ABL1 tyrosine kinase. The novel BCR-ABL1 protein has a deregulated, constitutively activated tyrosine kinase activity that drives the cancer phenotype. A novel class of therapeutic agents, tyrosine kinase inhibitors (TKIs), have been developed that bind and

repress the activity of the ABL1 kinase domain (1). The first successful drug in this class, imatinib, significantly represses the leukemic clone without the typical toxicity of conventional nonspecific anticancer drugs, and has become the consensus first-line therapy for CML (2–6). The vast majority of imatinib-treated chronic-phase CML patients undergo a complete hematologic and cytogenetic response (3, 7, 8). However, despite these excellent initial responses, a minority of patients subsequently experience secondary (acquired) imatinib resistance as evidenced by a loss of their complete cytogenetic response (CCR). Among patients with secondary imatinib resistance, up to half harbor acquired mutations in the BCR-ABL1 kinase domain that abrogate imatinib binding to its target (9–12). Currently, over 70 mutations within the BCR-ABL1 tyrosine kinase domain have been identified (10, 13, 14). As most, but not all, of these KD mutants are sensitive to inhibition by second-generation TKIs, dasatinib and nilotinib, treatment with these second-line agents is often quite effective after imatinib failure (15). Notable exceptions, however, include some mutations that are particularly insensitive to nilotinib (Y253H, E255K/V, F359V/C), to dasatinib (F317L, V299L), or to all approved TKIs (T315I) (15). Given that the discovery of one of these mutations with dasatinib and/or nilotinib resistance would predict poor efficacy with that agent, the exact identification of BCR-ABL1 KD mutations has become a consensus method for evaluating putative drug resistance in CML (4, 5, 14). Although a variety of mutation-scanning methods can be used to detect KD mutations, the majority of labs undertake BCR-ABL1 mutation analysis by direct DNA sequencing of the entire KD (14). The unbiased direct sequencing approach allows identification of patients currently on imatinib that would benefit (or not) from second-generation TKIs. Additionally, sequencing identifies patients with the T315I mutation who may benefit from new experimental TKIs such as ponatinib (16). Other more toxic alternative therapies (e.g., stem cell transplant) could then be reserved only for those patients most likely to benefit from the additional risk. Our laboratory's protocol for direct DNA sequencing of the BCR-ABL1 kinase domain is detailed below.

2 Materials

2.1 Cell Lysis

1. Samples for BCR-ABL kinase domain sequencing can be peripheral blood (drawn into EDTA or citrate tubes; minimum 5 ml) or bone marrow (minimum 2 ml in heparinized syringe, transferred to EDTA tube). The sample should arrive in the lab (room temperature transportation) no later than 24 h after being drawn. For the typical patient with quantitative BCR-ABL

RQ-PCR samples being evaluated in the same lab, residual RNA prepared for the same sample's RQ-PCR assay can be used (stored at -80°C).

2. Erythrocyte lysis buffer (Buffer EL Qiagen, Valencia, CA).
3. White cell lysis buffer ((Bottle #3) MagNA Pure LC mRNA HS Kit (Roche, Indianapolis, IN)).

2.2 Automated Isolation of mRNA Using Roche MagNA Pure Instrument

1. MagNA Pure LC mRNA HS Kit (Roche, Indianapolis, IN).
2. MagNA Pure consumables, including MagNA Pure small, medium, and large reagent tubs; large and small reaction tips; processing cartridges; and sample cartridges.
3. MagNa Pure LC 2.0 (Roche, Indianapolis, IN) automated nucleic acid extraction instrument.

2.3 Reverse Transcription of RNA to cDNA

1. Superscript II reverse transcriptase enzyme (Invitrogen, Carlsbad, CA).
2. 5× RT Buffer (included in Superscript II package).
3. 100 mM dithiothreitol (DTT) (included in Superscript II package).
4. Random primers p(dn)₆ (Roche Diagnostics, Indianapolis, IN) dissolved in 1 ml of RNase-free PCR grade H₂O (1 mM stock).
5. PCR nucleotide mix (Roche Diagnostics, Indianapolis, IN); each at a concentration of 10 mM.
6. Protector RNase Inhibitor, 40 U/μl (Roche Diagnostics, Indianapolis, IN).
7. MJ Research PTC-200 Peltier Thermal Cycler.

2.4 PCR

1. Primers:
 Forward BCR gene: ~40–60 bp's upstream of B2A2 fusion junction.
 BCR-ABL-1st-step-F (1F): 5' c tga cca act cgt gtg tga aac t 3' (bcr gene, Accession # Y00661; Nucleotide # 3240).
 Reverse ABL1 gene: ends ~8 bp's downstream from Glu-Thr-Met C terminus of kinase domain.
 BCR-ABL-1st-step-R: 5' cc act tcg tct gag ata ctg gat 3' (ABL1 gene: Accession # NM_005157; Nucleotide # 1499).
 Forward E1A2 BCR gene: starts ~200 bp's upstream of E1/A2 "minor" breakpoint (see Note 1).
 BCR-ABL-1st-F-e1a2 (1Fe1a2): 5' acc gca tgt tcc ggg aca aaa g 3' (bcr gene: Accession # Y00661; Nucleotide # 1675).
2. 10× Buffer 1 (1.75 mM Mg²⁺) (included in Roche® Expand Long Template PCR System (Roche Diagnostics, Indianapolis, IN)).

3. Dimethylsulfoxide.
4. dNTPs (15 mM each).
5. Expand Long Template Taq (5 U/μl) (included in Roche® Expand Long Template PCR System (Roche Diagnostics, Indianapolis, IN).
6. MJ Research PTC-200 Peltier Thermal Cycler.

2.5 PCR Product Visualization and Purification

1. 1% DNA agarose gel.
2. Low Mass DNA Ladder (Invitrogen, Carlsbad, CA).
3. 6× Type I gel loading dye.
4. ExoSap-IT (Affymetrix, Santa Clara, CA) (a mixture of two hydrolytic enzymes, exonuclease I and shrimp alkaline phosphatase).

2.6 Sequencing

1. BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA).
2. 5× Sequencing Buffer (included in BigDye kit).
3. Primers for ABL1 kinase domain sequencing (F=forward strand; R=reverse strand):
 BCR-ABL-2nd-step-F [2F]: 5'cgc aac aag ccc act gtc t 3' (*ABL1* gene: Accession # NM_005157; Nucleotide # 661).
 BCR-ABL-1st-step-R [1R]: 5' cc act tcg tct gag ata ctg gat 3' (*ABL1* gene: Accession # NM_005157; Nucleotide # 1500) (see Note 2).
 Seq-BCRABL-MF [MF]: 5'cat cca cag aga tct tgc tgc c 3' (*ABL1* gene: Accession # NM_005157; Nucleotide # 1080).
 Seq-BCRABL-MR [MR]: 5' ccc cta cca ggc agt ttc g 3' (*ABL1* gene: Accession # NM_005157; Nucleotide # 1102).
 ABL-R6 [Hein]: 5' aga act tgt tgt agg cca 3' (*ABL1* gene: Accession # NM_005157; Nucleotide # 1235).
 ABL-3F: 5' tga cag ggg aca cct aca ca 3' (*ABL1* gene: NM_005157; Nucleotide # between 1141 and 1320).
4. MJ Research PTC-200 Peltier Thermal Cycler.
5. DyeEx 96 Kit (Qiagen, Valencia, CA).
6. Tabletop microplate centrifuge.
7. 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA).
8. Hi-Di formamide (Applied Biosystems, Carlsbad, CA).
9. ABI 3130 septa, black plate holders, 96-well plates.
10. SeqScape sequence analysis software (Applied Biosystems, Carlsbad, CA).

3 Methods

CML patients receiving imatinib therapy are routinely monitored every 3–6 months with serial BCR-ABL1 RQ-PCR transcript levels. A rise of BCR-ABL1 RNA above a 2.6-fold optimized cutoff has been shown to be associated with a significantly increased risk of harboring a concomitant KD mutation (8). Thus, when a patient's transcript level rises, the same sample is often referred for DNA sequencing. If a mutant clone other than T315I is detected, the patient often becomes a candidate for treatment with a second-generation TKI. If the T315I mutation is detected, then, if available, a novel TKI or alternative non-pharmacologic measures such as stem transplantation are considered. Our method for quantitating BCR-ABL1 transcripts in peripheral blood or bone marrow is described in an accompanying chapter of this book. Here we detail our method for DNA sequencing the BCR-ABL1 KD from these same samples.

3.1 *Lysis of Cells from Blood or Bone Marrow*

1. Count cells from the raw blood or bone marrow sample using an automated hematology cell counter. Alternatively, a manual cell count can be performed. Use the total leukocyte count to determine the blood or bone marrow volume necessary to achieve a total of 5.0×10^7 white blood cells.
2. After calculating the volume of bone marrow or blood to be extracted (50 million cells), transfer that volume to an appropriate tube, and add a 2× volume of Qiagen Buffer EL (erythrocyte lysis) to the sample.
3. Place the tubes on a rocking platform or gyrotory shaker for 10 min at room temperature to lyse the red blood cells. Alternatively, manually invert the sample periodically for 10 min.
4. Centrifuge the tubes at $980 \times g$ for 5 min in a tabletop centrifuge (with the brake off) at room temperature. After centrifuging, a white cell pellet should be visible at the bottom of the tube (corresponding to 50 million cells).
5. With a sterile pipette, carefully remove the supernatant, or pour off supernatant into a waste container, but do not disturb the pellet.
6. To eliminate any residual red blood cells that may later interfere with PCR, add an additional 5–10 ml of red cell lysis buffer (EL) to the white cell pellet, resuspend the cells, and transfer to a 15 ml conical tube.
7. Spin the sample again at $980 \times g$ for 5 min. After centrifuging, a clean white cell pellet should be visible at the bottom of the tube.
8. With a sterile pipette, carefully remove the supernatant, or pour off supernatant into a waste container, but do not disturb the pellet.

9. Based on the number of cells extracted, calculate the volume of white blood cell lysis buffer (bottle #3 in MagNA Pure mRNA HS Isolation Kit) needed to achieve a final concentration of 1.0×10^7 white cells per ml, up to a total lysis buffer volume of 5 ml for 50 million cells.
10. After removal of the red cell lysis supernatant, add the appropriate volume of WBC lysis buffer to the WBC pellet.
11. Thoroughly resuspend the white cell pellet in the lysis buffer, by pipetting up and down several times.
12. Vortex the lysate briefly, and allow the lysate to rest for 5 min.
13. After 5 min, vortex the lysate again.
14. The lysed sample may now be put directly onto the MagNA Pure for mRNA isolation, or may be frozen at -70°C for mRNA isolation at a later time.

3.2 Automated Isolation of mRNA Using Roche MagNA Pure Instrument

1. If lysates have been frozen, allow to thaw along with a 10^{-4} control (1:10,000 dilution of K562 cells into a BCR-ABL-negative cell line, e.g., HeLa cells).
2. Programming the instrument.
 - (a) Select “Start Program” on the MagNA Pure.
 - (b) Select “Sample Ordering.”
 - (c) Under *Sample Protocol*, select “mRNA 1 cells.blk.”
 - (d) Under *Sample Volume*, type in the amount of cellular lysate you are starting with. This volume must be the same for all samples extracted in a given run, and will usually be $300\ \mu\text{l}$ (*usually three million cells, to reflect lysates frozen at ten million cells per ml*).
 - (e) Under *Elution Volume*, change $25\ \mu\text{l}$ to $50\ \mu\text{l}$. Do not choose a *Post-Elution Protocol*.
 - (f) Type in patient identifiers on “Sample Ordering” List. When the identifiers (sample numbers) have been input for each sample to be extracted, click on “Start Batch.” You will see a screen which indicates the volumes of all the reagents and types of consumables needed to complete the isolation process.
3. Preparing reagents and disposables.
 - (a) Remove the metal tub rack from the instrument and place the appropriate sized reagent tubs in the tub rack.
 - (b) Fill the reagent tubs with the given volumes of reagents (all reagents are part of the *MagNA Pure mRNA HS Kit*). Be sure to put the reagent tubs in the correct order, and skip positions as indicated.
 - (c) Return the tub rack to the instrument stage and close the lockbar.

- (d) Place *sample processing cartridges* in the heating and cooling blocks.
 - (e) Place the appropriate number of *processing cartridges* in the correct positions, as indicated on the screen.
 - (f) Insert the necessary number of *large tips* (blue) and *small tips* (yellow) into the appropriate positions.
 - (g) Position a solid waste bag on the disposal chute and hold it in place with the magnet provided.
 - (h) Pipette the correct volume (300 µl) of cell lysate for each sample into the appropriate wells of a *sample cartridge* and place it in the instrument. Close the instrument door and click on the icon for each reagent and disposable that has been placed in the instrument.
 - (i) Once all icons have been selected, an “OK” button will be visible on the screen. Click on the “OK” button.
 - (j) The MagNA Pure will begin by slowly moving the robotic arm across the stage, to make sure that no obstacles are present. It will then begin extracting samples, eight at a time.
4. Aliquoting extracted mRNA upon program.
- (a) After the MagNA Pure instrument has completed the mRNA isolation process, mRNA samples will be in the sample cartridge in the cooling block of the instrument. Carefully aliquot the eluted mRNA (50 µl) into pre-labeled 1.5 ml tubes using pipettes designated for RNA use only.
 - (b) Place tubes of mRNA on ice if directly proceeding with reverse transcription, or store at -70°C for future use.
5. Dispose of consumables, and close out windows until the front screen appears. Select “Decontamination.” Next, select “Start Decontamination” from the “Actions” pull-down at the top of the window. Allow the UV decontamination of the MagNA Pure to proceed for 8 h (overnight).

3.3 Reverse Transcription

1. Start the following “RT” program on the thermocycler. Block will heat up to 65°C “forever.”
- “BCR” Program (for reverse transcription)*
- (a) 65°C “forever.”
 - (b) 25°C for 10 min.
 - (c) 42°C for 50 min.
 - (d) 70°C for 15 min.
 - (e) 4°C “forever.”
2. Place mRNA samples and RT reagents on ice to thaw. Include a positive and a negative control (see Note 3).

Table 1
Reverse transcription master mix

Reagent	Reaction (μ l)	Final concentration
5 \times RT Buffer ^a	4	1 \times
100 mM DTT ^a	2	10 mM
Roche Pd(N) ₆ (1 mM)	1	50 μ M
dNTPs (10 mM ea)	1	0.5 mM ea
SuperScript II (200 U/ μ l)	1	10 U/ μ l
Roche protector RNase inhibitor (40 U/ μ l)	1	2 U/ μ l
Total volume	10 μ l	

Total RT reaction = 20 μ l

^aComes with Superscript II

3. Aliquot 10 μ l of mRNA (from about 1.5 million cells) into appropriately labeled 0.5 ml tubes.
4. Place in thermal cycler to denature at 65°C and set timer for 10 min.
5. While denaturing, prepare an RT master mix as shown in Table 1 and place on ice.
6. After 10 min at 65°C, place tubes containing mRNA on ice; “proceed to next step” on thermal cycler, and press “pause,” which will decrease the temperature to 25°C and hold it there.
7. Add 10 μ l of master mix to tubes containing denatured mRNA (total RT reaction volume is 20 μ l). Mix by pipetting up and down.
8. Place tubes in thermal cycler and resume program.
9. After RT is completed, store cDNA at 4°C short term (24 h) or -20°C long term.

3.4 PCR

1. Begin the following program on the thermocycler. Pause program when temperature reaches 95°C.
“ABL-SEQP” Program
 - (a) 95°C for 2 min.
 - (b) 95°C for 20 s.
 - (c) 58°C for 30 s.
 - (d) 68°C for 90 s.
 - (e) Repeat steps b–d, 44 times.
 - (f) 68°C for 7 min.
 - (g) 4°C “forever.”

Table 2
PCR master mix

Reagent	Reaction (μl)	Final concentration
10× Buffer 1 ^a (1.75 mM Mg ²⁺)	5.0	1× w/175 μM Mg ²⁺
DMSO	5.0	
dNTPs (15 mM ea)	1.5	0.45 mM ea dNTP
Bcr-abl-1st-step-F primer (1F) 30 μM	1.0	0.6 μM
Bcr-abl-1st-step-R primer (1R) 30 μM ^b	1.0	0.6 μM
Expand Long Template Taq ^a 5 U/μl	0.75	0.075 U/μl
PCR grade H ₂ O	30.75	
Total volume	45	

Final PCR reaction volume = 50 μl

^aRoche® Expand Long Template PCR System

^bOr, try e1a2 “minor” breakpoint forward primer

2. Prepare PCR master mix as shown in Table 2 in 0.5 ml PCR tubes.
3. Aliquot 45 μl of master mix into 0.5 ml empty labeled tubes.
4. Add 5 μl cDNA to each PCR tube.
5. Place tubes in thermal cycler and resume program.
6. Place cDNA back into –20°C freezer for storage in case a repeat PCR is necessary.

3.5 Visualization of PCR Products

1. Prepare a 1% DNA agarose gel (see Note 4).
2. Load 4 μl of amplicon with 1 μl 6× Type I loading dye. For approximate quantification of product, load 4 μl of Low Mass DNA Ladder.
3. Run gel for approximately 20 min at 100 V.
4. Stain in ethidium bromide for 10 min and destain for 10 min.
5. Photograph the gel on a UV box.
6. Compare BCR-ABL1 RT-PCR product bands to the ladder (see Note 5). If sample bands (of the correct size) look to be approximately 80 ng or greater, go to Subheading 3.6 (purification/cleanup of PCR products). If the bands are weak or undetectable, and this correlates with an expectedly low-level BCR-ABL1 RNA (below ~0.3% on the international scale), then set up a nested PCR reaction, as in step Nested PCR, steps a–f. If the sample is known to have high-level BCR-ABL1 RNA and there is no visible amplicon, the presence of an alternative e1a2 “minor” translocation breakpoint is possible,

and the sample should be re-amplified using an E1/A2 forward primer, as in Minor Breakpoint E1/A2 PCR, steps a–c.

Nested PCR

- (a) Prepare same PCR master mix as above, using the same primers for the nested reaction as for the first-round PCR, as in Subheading 3.4 (PCR), steps 1–6. Be sure to include two negative controls: the negative control from the first round of PCR, and a new negative control (water).
- (b) Aliquot 45 μ l of master mix into 0.5 ml tubes in the main lab (amplicon-free).
- (c) Take the master mix to the PCR room to add 5 μ l of first-round PCR product.
- (d) Use the same program on the thermal cycler as for first-round PCR (see Subheading 3.4 PCR, step 1).
- (e) Load the product onto a test gel again to check for a nested PCR product.
- (f) If no band is visible after nested PCR, report out the case as “PCR failure” (either a technical failure or BCR-ABL1 RNA level too low to amplify for sequencing).

Minor Breakpoint E1/A2 PCR

- (a) Prepare the same PCR master mix as before, but substituting “Bcr-abl-1st-F-e1a2” primer (30 μ M stock) for primer 1F [see section 2.4 (PCR) for primer sequences].
- (b) Run the thermal cycler program as in section 3.4 (PCR).
- (c) Add 5 μ l of cDNA to each 50 μ l PCR reaction.

3.6 Purification/ Cleanup of PCR Products Using ExoSap-IT

1. To a new PCR tube, add 6 μ l of ExoSap-IT to 15 μ l of PCR product. Mix well.
2. Run samples on the thermocycler with the following program:
 - (a) 37°C for 15 min (to remove primers and dNTPs from PCR reaction).
 - (b) 80°C for 15 min (to inactivate the ExoSAP-IT enzymes).

3.7 Setting Up Sequencing Reaction

1. Set up six separate sequencing reactions (one for each of six different sequencing primers) for each PCR product template as shown in Table 3.
2. Place in the thermocycler with the following cycle sequencing program:
 - (a) 96°C for 1 min.
 - (b) 24 cycles of 96°C for 10 s.
 - (c) 50°C for 5 s.
 - (d) 60°C for 4 min.
 - (e) Hold at 4°C.

Table 3
Sequencing reaction master mix

BigDye 1.1 terminator ready mix	2 µl
5× Sequencing buffer	2 µl
3.2 µM Primer	1 µl
DNA (post ExoSap-IT PCR product)	3.5 µl
dH ₂ O	11.5 µl
Total volume	20 µl

3. At this point, samples can be stored at 4°C protected from light for up to 4 days.

**3.8 Removal of
Unincorporated Dye
Terminators from
Sequencing Reactions**

DyeEx spin columns are pre-hydrated gel filtration columns designed to remove unincorporated dye terminators from cycle sequencing reactions.

1. Set up Qiagen DyeEx plate (see Note 6) in blue collection tray. Remove the bottom foil, then the top foil for the number of samples needed.
2. Spin plate on blue (waste) collection tray to elute pre-hydration buffer for 3 min at 750×g in the tabletop microplate centrifuge.
3. Discard the flow-through and save the reusable waste collection tray.
4. Transfer the DyeEx plate on top of a clean 96-well plate that will collect the column flow-through for subsequent capillary electrophoresis.
5. Carefully load the entire sequencing reaction (20 µl) onto the DyeEx spin column resin bed (see Note 7).
6. Spin the plate stack for 3 min at 750×g in a microplate centrifuge to elute the sample. The gel filtration column will retain unincorporated dye terminators from the sequencing reactions. Final elution volume will be approximately 20 µl.

**3.9 ABI 3130
Capillary
Electrophoresis
Using 36 cm Array**

1. Put the uncovered 96-well plate in the designated heat block and heat at 70°C until samples are completely evaporated (about 45 min).
2. Resuspend the dried samples in 20 µl of Hi-Di formamide.
3. Gently pipette up and down to resuspend, taking care not to introduce air bubbles (see Note 8).
4. Cover the plate with a gray septum (gasket) and denature samples at 95°C for 5 min.

5. Place the plate in a black plate holder, put on a white lid (make sure holes in lid and septum are aligned), and place this “sandwich” onto the ABI 3130 instrument.
6. On the ABI instrument, replace the anode buffer in the anode buffer jar and reservoir #1 with fresh buffer. Replace the water in reservoirs #2–4 in the instrument.
7. Set up a plate record in the ABI data collection software, link to plate, and click on \Rightarrow (run).
8. See Note 9 for suggested naming convention for sequencing samples.

3.10 Sequencing Data Analysis

1. Open ABI *Sequencing analysis* program.
2. Under *File*, click *Add Samples*.
3. Locate previously saved data files, click (to add the file to the analysis queue), and then click OK.
4. Click on \Rightarrow (run) at top of screen; this will begin basecalling (by sequence analysis software).
5. Next, click to check boxes in *P* column (for print) and click on \Rightarrow (run) once more. Data (electropherograms) will now be printed.
6. Under *File*, click *Save all samples*.
7. Acceptable run criteria:
 - (a) Sensitivity control (also positive mutation control; BCR-ABL RNA ratio $\sim 0.1\%$) must amplify and yield expected sequence.
 - (b) Positive mutation control must show the expected mutation (and no others).
 - (c) Negative control should not show any product on test gel (not sequenced).
8. The raw ABI-generated sequencing data can be analyzed a number of different ways (even manually), depending on the lab’s needs and resources. Software for analysis of raw DNA sequencing data is available from several commercial vendors. In our lab, we use Seqscape® software from ABI, which we have internally validated as an accurate and sensitive DNA sequencing data analysis package. With Seqscape, the experimental data can be automatically compared to a reference *BCR-ABL1* sequence from Genbank, and automated reports can be generated detailing the location of putative mutations and the “quality” of the mutation determination. Seqscape’s analysis settings can also be user adjusted to alter the “sensitivity” for detecting “mixed bases” when low-level variants are present. This is an important parameter for finding BCR-ABL1 kinase domain mutations that may be an evolving cause of resistance to kinase inhibitor therapy. In our lab, we set

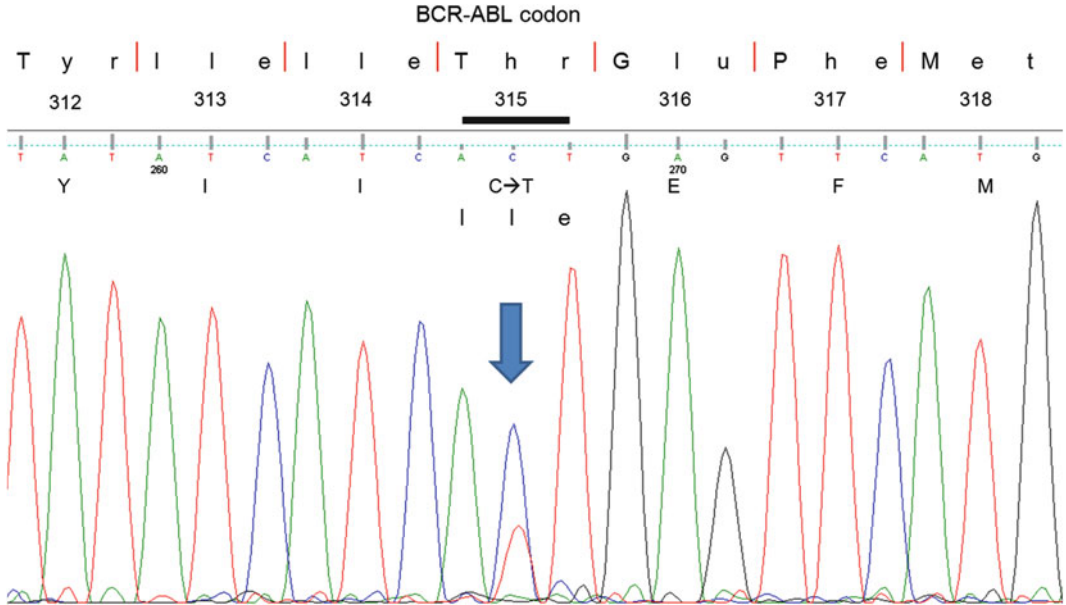


Fig. 1 A representative DNA sequencing electropherogram showing a T315I point mutation in the BCR-ABL kinase domain. The *arrow* indicates the location of a C to T single nucleotide mutation which changes a wild-type threonine amino acid (ACT) to isoleucine (ATT). The mutation appears to be expressed in only a minority of cells given the increased signal of the wild-type C nucleotide (*blue*) compared to the mutant T nucleotide (*red*)

Seqscape’s “mixed base” analysis setting to 15%, which allows us to typically detect variant clones that are present at a level of approximately 15–20% in a background of wild-type BCR-ABL1. The consequence of this low mutation detection threshold setting is an increase in the number of “false-positive” mutation calls that must then be evaluated manually to discern whether or not they are “real.” See Fig. 1 for a representative DNA sequencing electropherogram from a patient with a T315I point mutation. Figure 2 shows the frequency and heterogeneous distribution of mutations in the BCR-ABL1 kinase domain detected over an 8-year period at our institution.

4 Notes

1. The vast majority of patients have a p210 “major” bcr-abl breakpoint downstream of BCR exons b2 or b3. The “1F” forward primer will work in these cases. If the 1F/1R primer pair does not yield an expected 1.5–1.6 kb amplicon (and the BCR-ABL1 RNA ratio is above 0.02%), a “minor” c1a2 breakpoint is suspected—requiring the alternative 1Fe1a2 forward primer. A small minority of patients will have a p190 “minor”

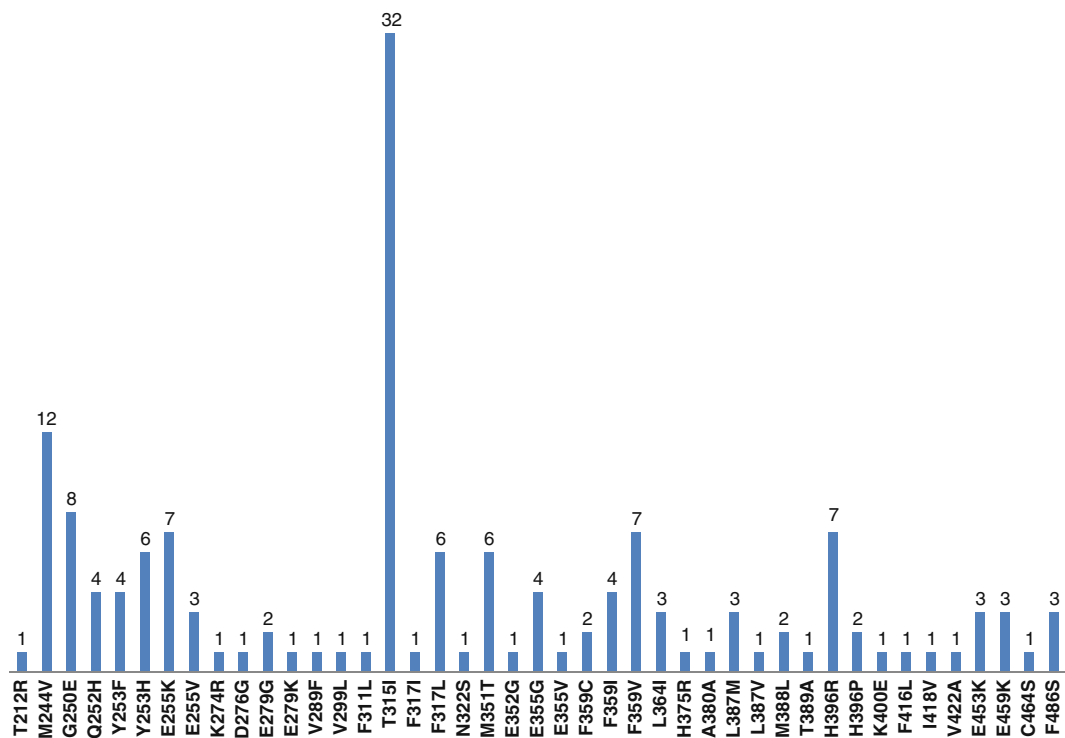


Fig. 2 The frequency of BCR-ABL kinase domain mutations in 110 leukemic patients followed at our institution. The total number of mutations sums to 153 indicative of the presence of multiple mutations in some patients. The X-axis depicts the BCR-ABL mutation locus using amino acid numbering as per Genbank accession number NM_005157. The Y-axis depicts the number of patients with each mutation. In these 110 patients, 43 distinct mutations have been detected at 34 different codons

- breakpoint downstream of bcr exon c1. The exact breakpoint may or may not be known prior to sequencing.
2. The same reverse primer was used to generate the 1.5–1.6 kb BCR-ABL1 amplicon for sequencing. BCR-ABL1 RNA level must be above 0.02% (BCR-ABL1/G6PDH) to proceed with ABL1 sequencing. Levels below that cutoff will usually be insufficient to generate enough of the long BCR-ABL1 PCR product for successful sequencing.
 3. For the positive mutant control, we rotate through various patient specimens previously identified as containing a mutation. For the negative control, we use water.
 4. Use aerosol tips to prevent contamination when loading product onto gel.
 5. PCR product (1F and 1R primers) is 1,579 bp for b3a2 and 1,504 bp for b2a2.

6. For small numbers of sequencing samples, individual DyeEx columns can be used in place of the plate method. This method follows the same procedure as above.
7. Do not touch the sides of the column or the resin bed with the tip.
8. Any bubbles that are inadvertently introduced may be removed with a pipette tip.
9. Name as sample number_primer name (e.g., n06-001014_2F, n06-001014_1R).

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