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Research Article

## A customized mass array panel for *BCR::ABL1* tyrosine kinase domain mutation screening in chronic myeloid leukemia

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ABSTRACT

**Introduction:** The therapeutic strategy and management of chronic myeloid leukemia (CML) have rapidly improved with the discovery of effective tyrosine kinase inhibitors (TKIs) to target *BCR::ABL1* oncoprotein. However, nearly 30% of patients develop TKI resistance due to acquired mutations on the tyrosine kinase domain (TKD) of *BCR::ABL1*.

**Methods:** We customized a mass array panel initially intended to detect and monitor the mutational burden of hotspot *BCR::ABL1* TKD mutations accumulated in our database, including key mutations recently recommended by European LeukemiaNet. Additionally, we extended the feasibility of using the assay panel for the molecular classification of myeloproliferative neoplasms (MPNs) by incorporating primer sets specific for analyzing JAK2 V617F, MPL 515 K/L, and CALR types 1 and 2.

**Results:** We found that the developed mass array panel was superior for detecting and monitoring clinically significant *BCR::ABL1* TKD mutations, especially in cases with low mutational burden and harboring compound/polyclonal mutations, compared with direct sequencing. Moreover, our customized mass array panel detected common genetic alterations in MPNs, and the findings were consistent with those of other comparable assays available in our laboratory.

**Conclusions:** Our customized mass array panel was practicably used as a routine robust assay for screening and monitoring *BCR::ABL1* TKD mutations in patients with CML undergoing TKI treatment and feasible for analyzing common genetic mutations in MPNs.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm characterized by the rapid proliferation of malignant myeloid cells at all stages of differentiation. With an incidence of 1–2 cases per 100,000

adults, it accounts for approximately 15% of new cases of adult leukemia [1]. A genetic translocation between chromosomes 9 and 22, t(9;22)(q34;q11.2), resulting in the Philadelphia chromosome (Ph chromosome), is recognized as the central pathogenesis of this disease. The Ph chromosome produces a *BCR::ABL1* oncoprotein, which constitutively

**Abbreviations:** ABL1, ABL proto-oncogene 1; BCR, breakpoint cluster region; CALR, calreticulin; DNMT3A, DNA methyltransferase 3 alpha; EMA, European medicines agency; ET, essential thrombocythemia; FDA, food and drug administration; FISH, fluorescence in situ hybridization; IDH1, Isocitrate dehydrogenase 1; JAK2, Janus kinase 2; JUN, JUN (ju-nana) proto-oncogene; LOD, limit of detection; MRD, measurable residual disease; MPNs, myeloproliferative neoplasms; MPL, myeloproliferative leukemia; MYC, myelocytomatosis; NGS, next-generation sequencing; PV, polycythemia vera; PMF, primary myelofibrosis; RAF, rapidly accelerated fibrosarcoma; RAS, Rat sarcoma virus; SETBP1, SET Binding Protein 1; STAT, signal transducer and activator of transcription; TKD, tyrosine kinase domain; TKIs, tyrosine kinase inhibitors.

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activates several tyrosine kinase pathways, including STAT, RAS, RAF, MYC and JUN [2–5], promoting hematopoietic stem cell transformation and initiating malignant leukemic cells [6].

Since the discovery of small-molecule tyrosine kinase inhibitors (TKIs) targeting the BCR::ABL1 oncoprotein, CML treatment has dramatically improved overall survival and quality of life for patients with CML [7–10]. Consequently, the 10-year overall survival rate of CML patients is approximately 80%–90% [1,11]. TKIs specifically block the interaction of BCR::ABL1 oncoprotein with adenosine triphosphate (ATP), thus interfering with cellular signaling and inhibiting proliferation while allowing apoptosis of the CML clone [10,12]. Five TKIs are commercially available and approved for use in treating CML: imatinib, dasatinib, nilotinib, bosutinib and ponatinib [12]. The Food and Drug Administration (FDA) and European Medicines Agency (EMA) have approved the first four of these for first-line treatment of CML with different suggestions such as stage of CML at diagnosis, doses and reimbursements [13]. Despite the dramatic improvement in treatment outcomes over the past decade with the development of new TKIs, approximately 20%–30% of patients become resistant to TKIs, leading to treatment failure and disease relapse [14–15].

Two main mechanisms are involved in the establishment of TKI resistance in patients with CML: BCR::ABL1-dependent and BCR::ABL1-independent ones [16–17]. BCR::ABL1-dependent mechanisms are known as the primary cause of TKI resistance, which mainly occurs via the acquisition of mutations in the tyrosine kinase domain (TKD) of the BCR::ABL1 fusion protein. Meanwhile, BCR::ABL1-independent mechanisms, including *BCR::ABL1* amplification and clonal evolution of CML clones, have been reported in CML with TKI resistance, albeit at lower frequencies [18–20]. BCR::ABL1-independent mechanisms can also consist of the impairment of specific TKI transporters (influx and efflux transporters), an inappropriate concentration of TKIs, dysregulation of alternative signaling pathways of CML cells, and epigenetics [21–28]. Recently, there has been evidence indicating that mutations in genes involved in the tumorigenesis of myeloid leukemia, such as *DNMT3A* and *ASXL1*, *IDH1*, and *SETBP1*, are associated with the resistance to TKIs therapy in CML without *BCR::ABL1* kinase domain mutations [29–30]. Additionally, evidence indicates that key driving mutations frequently identified in MPNs, including *JAK2* V617F or *CALR*, are concomitantly positive in patients with CML, which may contribute to CML's distinct phenotypes and involvement in the establishment of TKI resistance. Concurrent *BCR::ABL1* translocation and *JAK2* V617F mutation are rarely identified in atypical MPNs and both primary and secondary CML [31]. Furthermore, CML patients with the co-existence of *BCR::ABL1* translocation and *JAK2* V617F mutation exhibit prolonged persistent high *BCR::ABL1* mRNA levels during TKIs therapy [32]. Besides concurrent *BCR::ABL1* and *JAK2* V617F positivity in atypical MPNs, there has been a wave of evidence indicating that *CALR* mutations occurred in cases of *BCR::ABL1* positive CML [33]. *CALR* mutation (predominantly identified in the original clone of *BCR::ABL1* positive CML patient) might contribute to the persistence of MPN phenotypes regardless of BCR::ABL1 mRNA level during TKIs treatment [34]. Moreover, atypical MPNs who were positive for both *BCR::ABL1* and *CALR* mutations exhibited a response to imatinib treatment irrespective of the *CALR* mutation burden. Additionally, CML clones with *BCR::ABL1* positive/*CALR* negative had a superior response to imatinib than those harboring *BCR::ABL1* negative/*CALR* positive [35]. These data further highlight the importance of combining clinical and genetic data for classifying atypical MPNs, especially in cases with co-existing CML and MPN, which might be necessary for simultaneous identification of *BCR::ABL1* translocation and common genetic alterations in classical MPN, including *JAK2* V617F, *CALR*, and *MPL* W515K/L.

To date, acquired mutations of TKD in the *BCR::ABL1* coding sequence have been recognized as a significant cause of TKI resistance in CML. TKD mutations have been observed in approximately 50% of CML patients who do not respond to TKIs and exhibit disease progression [36–40]. To date, around 100 TKD mutation patterns affecting over 50

amino acid residues in the BCR::ABL1 protein have been identified in CML patients undergoing TKI therapy [12,41]. Common *BCR::ABL1* TKD mutations include M244 (nearby phosphate-binding loop: P loop), L248, G250, Q252, Y253, E255 (P loop), D276, E279 (c-helix), T315, F317 (gatekeeper residue), M351, F359 (SH2 contact and C-lobe), and H396 (activation loop) [30,42]. Notably, T315I mutation has been shown to be associated with resistance to all TKIs approved for frontline therapy (multiple TKI resistance) [36]. Recently, the ELN guidelines for the management of CML recommended therapeutic options for approved TKIs in the case of *BCR::ABL1* resistance mutations, specifically, T315I, F317L/V/I/C, T315A, V299L, Y253H, E255V/K, and F359V/I/C mutations [13]. While there have been recent improvements in therapeutic options using available TKIs for CML patients resistant to frontline therapy, various patients developed compound/polyclonal mutations (two or more mutations observed in a single patient) after sequentially taking TKIs, resulting in no response to several or all administered TKIs [30,43–45]. These findings further highlight the importance of molecular techniques in investigating *BCR::ABL1* TKD mutations in patients with CML undergoing TKI therapy.

Along with the detection of minimal residual disease (MRD; now referred to as “measurable residual disease”) by quantitative monitoring of BCR::ABL1 mRNA transcript, the analysis of *BCR::ABL1* TKD mutations is critical for managing CML. Several assays have been used for TKD mutation analysis, which vary in their performance, such as their sensitivity, specificity, rapidity, and cost-effectiveness. Direct sequencing performed on the product of reverse-transcription PCR (RT-PCR) specific to the amplification of BCR::ABL1 mRNA has been commonly used in many laboratories worldwide [14,46–47]. Although this technique can detect all mutations spanning the TKD domain, it has lower sensitivity and a longer turnaround time, and is more laborious than other available techniques, such as real-time PCR and droplet digital PCR (dd-PCR). Recently, several studies have demonstrated the application of next-generation sequencing (NGS) techniques, which are superior for the deep molecular early identification and monitoring of single and compound/polyclonal *BCR::ABL1* TKD mutations in CML [48–53]. However, NGS is only available in some laboratories and is difficult to standardize. We recently reported practical laboratory investigation tools for managing patients with CML and established a domestic TKD mutational database of CML patients [54]. Moreover, we have developed a mass array technique that is a straightforward and robust assay for screening *TP53* hotspot mutations in patients with acute myeloid leukemia (AML) [55]. In this report, we present customization of the mass array technique for the simultaneous detection of *BCR::ABL1* TKD mutations in patients with CML based on our TKD mutational database and recent ELN guidelines for the management of CML. Additionally, we improved our mass array panel by including primer sets specific for common genetic alterations in myeloproliferative neoplasms, including *JAK2* V617F, *MPL* 515K/L, and *CALR* types 1 and 2.

## Materials and methods

### Samples

Twenty-eight RNA samples derived from CML patients who underwent TKI therapy were included in this study. The *BCR::ABL1* TKD mutation status of these samples was recorded based on direct sequencing. Total RNA was isolated from peripheral blood or bone marrow samples using QIAzol Lysis Reagent (QIAGEN, Hilden, Germany) and purified with Direct-zol™ RNA Miniprep (Zymo Research, USA), in accordance with the manufacturer's instructions. The concentration of RNA was subsequently measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). This work was approved by the Ethics Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand, and followed the principles of the Declaration of Helsinki (ID MURA2019/90).

## Direct sequencing analysis

*BCR::ABL* kinase domain mutation analysis was performed by Sanger sequencing using a previously reported protocol [54]. Briefly, cDNA was generated from 1 µg of total RNA using the SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA). First-round PCR was used to amplify the *BCR::ABL1* fusion transcript, and the *ABL1* kinase domain was subsequently amplified in second-round PCR. The optimal conditions for both rounds of PCR were as follows: initial PCR at 95 °C for 10 min; 40 cycles of 94 °C for 1 min, 68 °C for 1 min, and 72 °C for 2 min; followed by a final extension at 72 °C for 10 min. After the PCR was complete, an 863-base-pair PCR product was analyzed by 2% agarose gel electrophoresis. This product was purified prior to the sequencing reaction using the ExoSAP-IT™ PCR Product Cleanup Reagent (Thermo Fisher Scientific, USA), in accordance with the manufacturer's instructions. The sequencing reaction was performed using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, USA) and 1.6 pmol of each sequencing primer. The amplification conditions were as follows: 96 °C for 1 min; 25 cycles of 96 °C for 10 s and 50 °C for 5 s; and then 60 °C for 4 min. The sequencing product was purified using the DyeEx 2.0 Spin Kit (QIAGEN, Germany) and subsequently sequenced with the ABI 3130 Genetic Analyzer (Applied Biosystems, USA), in accordance with the instruction protocol. Finally, the sequence was compared with the human *ABL1* reference sequence (GenBank accession number X16416.1) using SeqScape software version 4 (Applied Biosystems, USA).

A customized mass array panel for *BCR-ABL1* TKD mutation screening

In this work, we initially designed the MassArray® System (Agena Bioscience, CA, USA) to perform targeted, specific detection of *BCR::ABL1* TKD hotspot mutations in RNA samples derived from the blood and bone marrow of patients with CML during TKI therapy. The panel was customized based on our recently published data, primarily aimed at establishing a laboratory database of *BCR::ABL1* TKD mutations [54]. Moreover, we attempted to incorporate all of the *BCR::ABL1* TKD mutations listed in the current ELN guidelines for the management of CML [13]. Finally, we further improved the assay panel by adding several genetic hallmarks associated with the pathogenesis of common myeloproliferative neoplasms (MPNs) to expand its use in clinical practice.

Table 1

Customized mass array panel comprises clinically important, and hotspot mutations of *BCR::ABL1* TKD identified in CML patients during TKI therapy and common genetic alterations in MPNs.

Hotspot mutation	Nucleotide change	Reference SNP number	% Observed frequency [54]	Accession number		
<i>BCR::ABL1</i> TKD	248-274del	RNA splicing associated with L248V	-	0	NM_005157.6	
	L248V	CTG > GTG	rs121913455	0		
	G250E	GGG > GAG	rs121913453	2 (multiple mutations)		
	Q252H	CAG > CAC	rs121913458	0.4		
	Y253H	TAC > CAC	rs121913461	0.4		
	E255K	GAG > AAG	rs121913448	2		
	E255V	GAG > GTG	rs121913449	0		
	D276G	GAC > GGC	rs121913447	0.4		
	E279K	GAG > AAG	-	0		
	V299L	GTG > CTG	rs1057519771	0		
	T315A	ACT > GCT	rs1057519772	0		
	T315I	ACT > ATT	rs121913459	7		
	F317I	TTC > ATC	rs1057519773	0		
	F317L	TTC > CTC	rs1057519773	1		
	F317V	TTC > GTC	rs1057519773	0		
	F359C	TTC > TGC	rs1057519775	0		
	F359V	TTC > GTC	rs121913452	1		
	H396R	CAT > CGT	rs121913454	0		
	CALR type 1 (L367Tfs)	1092_1143del52	rs1555760738	10 ET [56]		NM_004343.3
	CALR type 2 (K385fs)	1154_1155insTTGTC	rs765476509	4 ET [56]		NM_004343.4
JAK2 V617F	GTC > TTC	rs77375493	95 PV, 75 ET, 25 PMF [57]	NM_004972.4		
MPL W515K	TGG > AAG	rs121913616	0 [56]	NM_005373.3		
MPL W515L	TGG > TTG	rs121913615	0 [56]	NM_005373.3		

Abberation: del, deletion; ins, insertion; ET, essential thrombocythemia; PV, polycythemia vera; PMF, primary myelofibrosis.

The list of *BCR::ABL1* TKD mutations and common genetic alterations in MPNs included in the developed mass array panel is shown in Table 1.

## Mass array for targeted genomic analysis

In this work, multiplex PCR was performed following our previous protocol for hotspot TP53 mutation analysis [55]. In brief, PCR amplification was performed using a polymerase chain reaction (PCR) primer mix (forward and reverse primers) with 100 nM MgCl<sub>2</sub> solution, 500 µM dNTP, 1x PCR buffer, 0.2 units/µL PCR enzyme, and 10–20 ng of DNA, making up a final volume of 5 µL. The PCR cycling conditions were as follows: 95 °C for 2 min; 45 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; and final extension at 72 °C for 5 min. PCR was subsequently performed to eliminate excess dNTPs by adding shrimp alkaline phosphatase (SAP). The SAP reaction mixture was as follows: 0.17 µL of 10x SAP buffer, 0.3 µL of SAP enzyme, and 1.53 µL of distilled water (HPLC grade). SAP thermal conditions were as follows: 37 °C for 40 min and 85 °C for 5 min. After the SAP reaction, the PCR product was added to the extension primer mix, which comprised primers specific for individual targets. A list of the PCR and extension primers is presented in Table 2. The second-round PCR reaction comprised 0.52–1.57 µM primer mix (extension primers), 0.222 × IPLEX® buffer, 0.222 × IPLEX® terminator mix, and 0.142 units/µL IPLEX® enzyme, making a final volume of 9 µL. The PCR thermal conditions of this step were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 52 °C for 5 s, and 80 °C for 5 s; and final extension at 72 °C for 3 min. The PCR product was then subjected to analysis by the MassArray® analyzer (Mass Spectrometry). Notably, because a tiny volume was dispensed, either an automated liquid handler or a manual dispenser was adopted by the manufacturer's certified technician. Finally, the genotypic data of target hotspot mutations were generated and reported using MassArray® Typer Software.

## Assay performance

We selected T315I as a target for validating the assay sensitivity because it was the most common *BCR::ABL1* TKD mutation identified in our database. It is also recognized as one of the most significant clinical mutations in the occurrence of treatment failure in patients undergoing TKI therapy. Furthermore, a homozygous T315I mutation was detected, representing 100% mutation burden in clinical samples, through direct

**Table 2**  
Amplification and extension primers for the mass array.

Hotspot mutation		PCR primers	Extension primers
<i>BCR::ABL1</i> TKD	(248-274del)	F-ACGTTGGATGGCACGGACATCACCATGAAG R-ACGTTGGATGAGACGCCCTTGCCCCACGTA	GACATCACCATGAAGCACAAG
	(L248V)	F-ACGTTGGATGGCACGGACATCACCATGAAG R-ACGTTGGATGGCTGTATTCTCCACACGC	CACCATGAAGCACAAG
	(G250E)	F-ACGTTGGATGGCTGTATTCTCCACAC R-ACGTTGGATGGCACGGACATCACCATGAAG	TCCCCGTA CTGGCCC
	(Q252H)	F-ACGTTGGATGCCGTGAGGCTGTATTCTTC R-ACGTTGGATGCATCACCATGAAGCACAAGC	ACACACGCCCTCGTACACCTCCCCGTA
	(Y253H)	F-ACGTTGGATGCCGTGAGGCTGTATTCTTC R-ACGTTGGATGCATCACCATGAAGCACAAGC	CACACGCCCTCGTACACCTCCCCGT
	(E255K)	F-ACGTTGGATGCCGTGAGGCTGTATTCTTC R-ACGTTGGATGCATCACCATGAAGCACAAGC	CGCCCTCGTACACCT
	(E255V)	F-ACGTTGGATGCCGTGAGGCTGTATTCTTC R-ACGTTGGATGCATCACCATGAAGCACAAGC	CTTCCACACGCCCTCGTACACC
	(D276G)	F-ACGTTGGATGTCAAGAACTCTTCCACCTCC R-ACGTTGGATGCCACTGAAAAGCACTTCCTG	TCCACCTCCATGGTG
	(E279K)	F-ACGTTGGATGAGGTTAGGGTGTITGATCTC R-ACGTTGGATGTTTCTCTCTCAGGAGGAC	TCTTCAAGAACTCTTCCACCT
	(V299L)	F-ACGTTGGATGAAAGTGCAGTCATGAAAGAG R-ACGTTGGATGAGACCTCTCTCAGAGCC	TCAAACACCCCTAACCTG
	(T315A)	F-ACGTTGGATGAGTCCTCGTTGTCTTGTGG R-ACGTTGGATGTCAAGTGTCCAGGAGGTTG	GCCCCGTTCTATATCATC
	(T315I)	F-ACGTTGGATGAGTCCTCGTTGTCTTGTGG R-ACGTTGGATGTCAAGTGTCCAGGAGGTTG	GTAGCCCCGTTCTATATCATCA
	(F317ILV)	F-ACGTTGGATGAGCCCCGTTCTATATCATC R-ACGTTGGATGTTGCACTCCCTCAGGTAGTC	CCGTTCTATATCATCACTGAG
	(F317L)	F-ACGTTGGATGTTCCCGTAGGTCATGAACTC R-ACGTTGGATGAGTCCTCGTTGTCTTGTGG	CCGTTCTATATCATCACTGAG
	(F359C)	F-ACGTTGGATGTTTTGTAAAAGGCTGCC R-ACGTTGGATGTACATGGCCACTCAGATCTC	GGCCCTACCTGTGGATG
	(F359V)	F-ACGTTGGATGTACATGGCCACTCAGATCTC R-ACGTTGGATGTTTTGTAAAAGGCTGCCG	GTACCTGGAGAAGAAAAAC
(H396R)	F-ACGTTGGATGGGTGCAGTCCATTTGATGGG R-ACGTTGGATGATTTGGCCTGAGCAGGTTG	GGAAGTGGCTCCAGCA	
<i>CALR</i> type 1		F-ACGTTGGATGTGAAGGACAAACAGGACGAG R-ACGTTGGATGTCTCATCTTTGTCTCTATC	GGACGAGGAGCAGAGG
<i>CALR</i> type 2		F-ACGTTGGATGAGACAAGAAACGCAAAGAGG R-ACGTTGGATGTCTCATCTTTGTCTCTATC	CAAAGAGGAGGAGGAGGAGGAGGACAA
<i>JAK2</i> V617F		F-ACGTTGGATGAGGCATTAGAAAGCCTGTAG R-ACGTTGGATGGCTTTCTCACAAGCATTGG	TAGTTTTACTTACTCTCGTCTCCACAGA
<i>MPL</i> W515K/L		F-ACGTTGGATGGCTCTGCATCTAGTCTGG R-ACGTTGGATGGTACCTGTAGTGTGCAGGA	CCGCTCTGGCCCTGCTGCTGCTGAGGT

sequencing. Therefore, this experiment did not require a synthetic DNA sequence of homozygous T315I mutation for sensitivity study. RNA with different serial dilutions of T315I positive and normal (75%, 50%, 25%, 10%, 5%, 2.5%, and 1% mutations) were performed and subjected to mass array analysis. The T315I mutation load of each RNA dilution was confirmed by standard direct sequencing, as described in the previous section.

To study the specificity of the assay, 28 RNA samples from CML patients with known *BCR::ABL1* TKD mutations and three samples of genomic DNA (gDNA) from MPN cases positive for each of *JAK2* V617F, *CALR* type I, and *CALR* type II were included. Moreover, gDNA isolated from bone marrow samples of patients with AML positive for *AML1::ETO*, *PML::RARA*, and *CBFB::MYH11* rearrangements was used as a negative control. For all samples, the mutation status was confirmed by direct sequencing (for *BCR::ABL1* TKD), AS-PCR (*JAK2* V617F), or PCR capillary electrophoresis (*CALR* types 1 and 2).

**Results**

The pre-customized mass array panel was initially designed based on the recurrent mutations spanning *BCR::ABL1* TKD observed in CML patients with TKI resistance in our laboratory. We also incorporated into this panel the key *BCR::ABL1* TKD mutations recommended in the ELN 2020 guidelines, including T315I, F317L/F317L/V/I/C, T315A, V299L, Y253H, E255V/K, and F359V. Moreover, we recently improved our previously designed mass array panel by adding to it well-established

genetic markers specific for the classification of classical MPNs (PV, ET, and PMF), including *JAK2* V617F, calreticulin (*CALR*) types 1 and 2, and *MPL* W515K/L. The list of *BCR::ABL1* TKD mutations in our customized mass array panel and common genetic alterations in classical MPNs used in the improved version is presented in Table 1. We further tested the performance of the generated mass array by considering direct sequencing, capillary electrophoresis PCR (CE-PCR), and allele-specific PCR (AS-PCR) as reference methods for *BCR::ABL1* TKD mutations and *MPL* W515K/L, *CALR* types 1 and 2, and *JAK2* V617F, respectively. Overall, 28 samples with CML and three samples with MPNs were included in this study. Notably, most CML cases were positive for *BCR::ABL1* TKD mutations with multiple mutation patterns. These were useful for exploring whether the customized panel could simultaneously detect multiple anomalies in a single run. For the specificity testing, we also included four samples: three AML cases positive for *AML1::ETO*, *CBFB::MYH11*, and *PML::RARA*, and one ALL case with *TEL::AML1*. We found concordance between the results of direct sequencing and mass array for detecting *JAK2* V617F and *CALR* types 1 and 2 (Table 3). However, in this work, no sample was positive for *MPL* W515K/L by both direct sequencing and mass array analyses. The accumulated data suggested the feasibility of our developed mass array panel for the molecular screening of common genetic alterations in classical MPNs.

Using the customized mass array panel for detecting *BCR::ABL1* TKD mutations, we found that 16 of 28 samples (57%) showed consistent data for the detection of *BCR::ABL1* TKD mutations. The mass array

**Table 3**

Direct sequencing and mass array data to detect *BCR::ABL1* TKD mutations from 28 samples of CML patients during TKI therapy. The mass array results for the detection of *JAK2* V617F and *CALR* types I and II were compared with AS-PCR and CE-PCR.

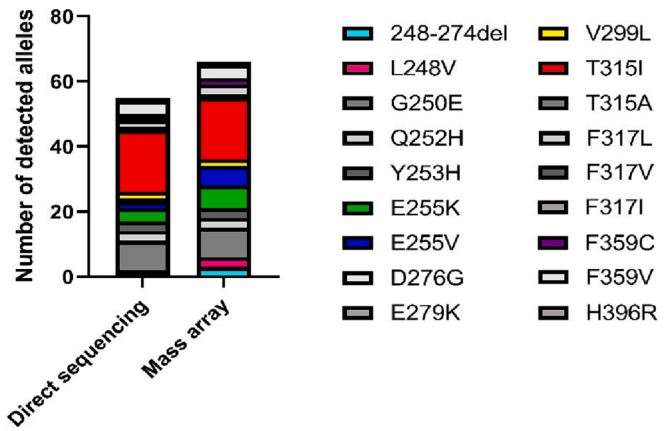
Sample	Direct sequencing	Mass array
CML001	E255K/T315I	E255K/T315I
CML002	G250E/Q252H /Y253H/ T315I/F359V	G250E/Q252H/Y253H/T315I/ F317L/F359V
CML003	T315I	T315I
CML004	E279K/H396R	H396R
CML005	G250E/T315A	G250E/T315A
CML006	L248V with del248-274/T315I	L248V with del248-274/T315I
CML007	Q252H/T315I	Q252H/T315I/F317L
CML008	G250E/V299L	G250E/V299L
CML009	G250E/Y253F/E255V	G250E/E255K/E255V/F359C
CML010	T315I/F359C	E255V/T315I/F359C
CML011	V299L/F317I/F317L	L248V with del248-274/V299L
CML012	T315I/F359V	T315I/F359V
CML013	G250E/F311I	L248V with del248-274/G250E
CML014	G250E/T315I/F359V	G250E/T315I/F359V
CML015	Y253H/T315I	Y253H/T315I
CML016	Q252H/T315I	Q252H/T315I
CML017	G250E/E255K/T315I	G250E/E255K/T315I
CML018	E255V/T315I	E255K/E255V/T315I
CML019	T315I	T315I
CML020	T315I	T315I
CML021	T315I	T315I
CML022	Y253H/F359V	Y253H/E255K/F359V
CML023	E255K	E255K
CML024	E255K/F317L	E255K/F317L
CML025	T315I	E255V/T315I
CML026	G250E/T315I	G250E/E255V/T315I
CML027	G250E/T315I	G250E/E255V/T315I
CML028	T315I	T315I
MPN001	JAK2 V617F*	JAK2V617F
MPN002	CALR Type1**	CALR Type1
MPN003	CALR Type2**	CALR Type2
AML001	Negative	Negative
AML002	Negative	Negative
AML003	Negative	Negative
ALL001	Negative	Negative

\* *JAK2* V617F analysis was performed by using allele-specific PCR (AS-PCR).  
\*\* *CALR* mutation screening was conducted using capillary electrophoresis PCR (CE-PCR).

**Table 4**

The number of each selected target of *BCR::ABL1* TKD mutations detected by direct sequencing and the customized mass array.

<i>BCR::ABL1</i> TKD mutation	Mutation detected by comparable methods among 28 CML samples	
	Direct sequencing	Mass array
248-247del	1	3
L248V	1	3
G250E	9	9
Q252H	3	3
Y253H	3	3
E255K	4	7
E255V	2	6
D276G	0	0
E279K	1	0
V299L	2	2
T315A	1	1
T315I	19	19
F317I	1	0
F317L	2	3
F317V	0	0
F359C	1	2
F359V	4	4
H396R	1	1



**Fig. 1.** Distribution of *BCR::ABL1* TKD mutation alleles was determined by direct sequencing and the customized mass array panel.

tended to be superior to direct sequencing in detecting more *BCR::ABL1* TKD mutations, especially E255K/V (Table 4 and Fig. 1), for which four cases remained undetected by direct sequencing but were positive when analyzed with the mass array panel (samples CML009, CML010, CML018, and CML022). Furthermore, the mass array showed advantages over direct sequencing in detecting L248V with concomitant harboring of the transcriptional variant 248–274del, as in CML011 and CML013 (Table 3). To assess the sensitivity and lower limit of detection (LOD) of the mass array panel, serial dilutions of RNA with T315I mutation (100% T315I detected by direct sequencing) in normal RNA were generated and subsequently analyzed using the customized mass array panel in triplicate and between runs. On the basis of visual evaluation, while direct sequencing could reliably distinguish T315I mutation at the LOD with 25% mutation, the mass array was superior in identifying T315I mutation with a lower mutational burden of 2.5% LOD (Figs. 2 and 3). This finding further suggested the value of the mass array for monitoring measurable minimal residual disease (MRD) in CML patients during TKI therapy. Additionally, simple linear regression analysis demonstrated that the established mass array exhibited remarkable linearity of the dilution of T315I mutational burden, and the measurable R<sup>2</sup> values of a mutant allele by mass array equaled 0.9680, 0.9503, and 0.9384, respectively (Fig. 4). Taking these findings together, the customized mass array panel showed advantages for application in the screening and MRD assessment of clinically important acquired *BCR-ABL1* TKD mutations in patients with CML undergoing TKI therapy. Furthermore, the assay could specifically detect recurrent genetic alterations, including *JAK2* V617F, *CALR* type 1 and type 2, and *MPL* W515K/L, in common MPNs.

In addition to studying assay performance, we compared the operational characteristics of the developed mass array panel with our current routine laboratory approaches for the analysis of *BCR::ABL1* TKD mutations, *JAK2* V617F, *CALR*, and *MPL* W515K/L. With its highest throughput, this version of our mass array could perform 96 analyses simultaneously, outperforming other available assays in our setting with a limited number of samples in a single run. Moreover, the developed mass array panel detected 23 genetic alterations (18 *BCR::ABL1* TKD hotspot mutations and 5 common mutations identified in MPNs) simultaneously, while the other available assays can analyze fewer mutations in a sample. Additionally, the mass array showed less operational complexity and a shorter turnaround time than the other techniques. Finally, the cost of the mass array analysis per sample was approximately £18, which is cheaper than comparable methods. The operational characteristics of our customized mass array panel and other comparable assays are listed in Table 5.

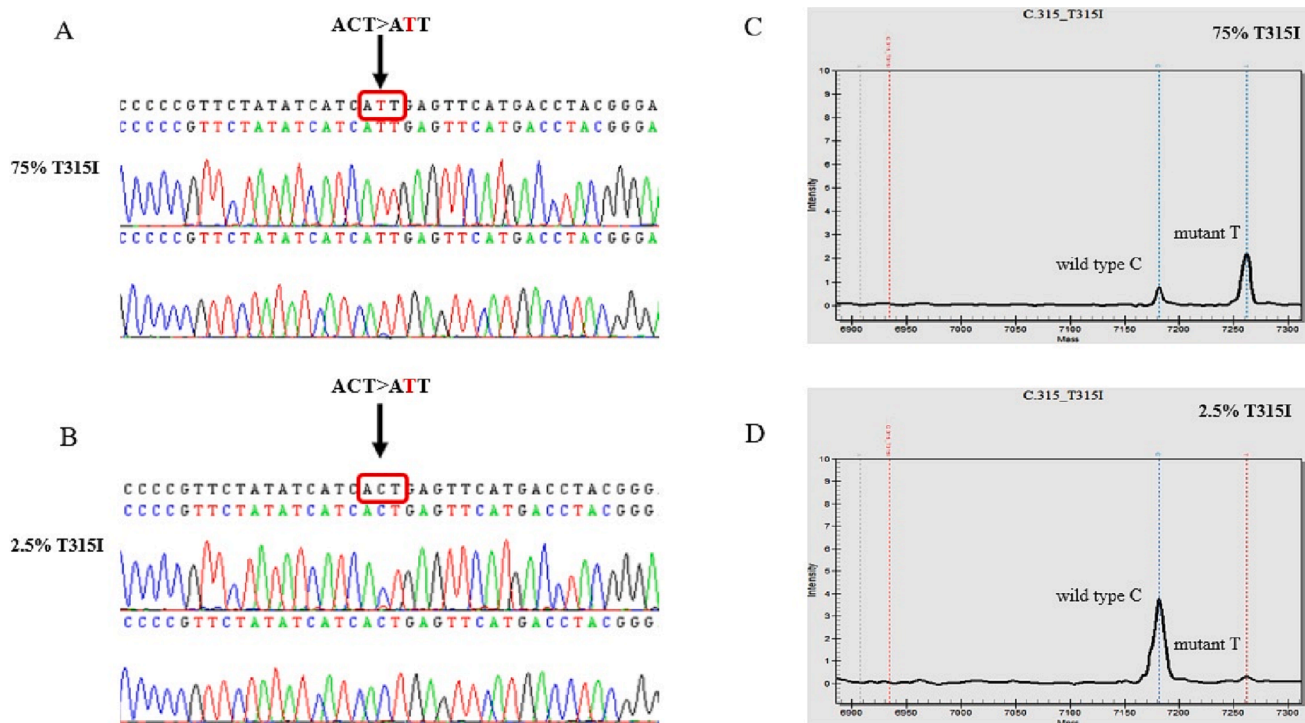


Fig. 2. Chromatogram demonstrating T315I mutational allele burden detected by direct sequencing and mass array. The upper A and C were conducted on 75% of a mutant allele, and the lower B and D were performed on 2.5% of the mutant allele.

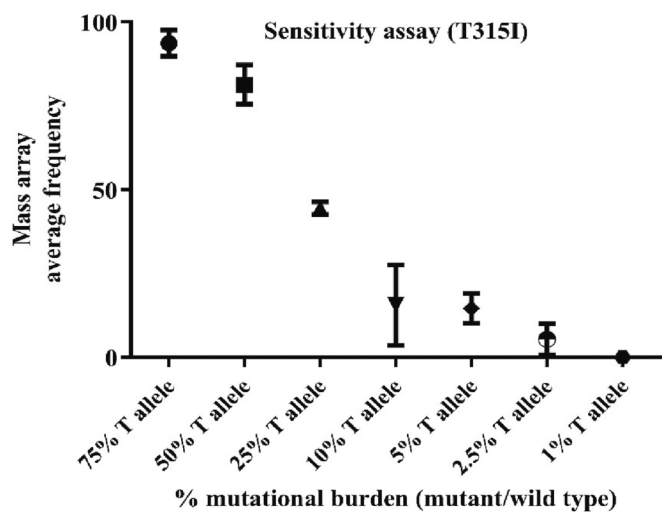


Fig. 3. Between run of replica experiments (triplicate) showing mass array data of sensitivity assay performed on a dilution of RNA from a patient with 100% *BCR::ABL1* T315I mutation detected by direct sequencing in normal RNA. The developed mass array panel detected *BCR::ABL1* T315I at a minimum mutational allele burden equaling 2.5% in normal RNA.

**Discussion**

Effective CML management comprises assessing the patient’s prognostic risk, and response to TKIs and other treatments, laboratory monitoring of the molecular response, supervision of treatment-free remission, managing adverse events, managing female patients who desire to retain their reproductive function, and reducing the financial burden [58]. Currently, the laboratory approach for effective management and molecular monitoring of CML during TKI therapy comprises cytogenetic analysis, FISH (optional, in an ambiguous or specific

scenario), quantitative PCR for *BCR::ABL1* mRNA, and *BCR::ABL1* TKD mutation analysis. In this report, we demonstrate the advantage of using mass array technology to detect clinically significant *BCR::ABL1* TKD mutations frequently identified in our laboratory and recommended in the recent ELN guidelines. Similar to other cohorts, we recently reported that acquired mutations of *BCR::ABL1* TKD are observed in approximately one-third of patients who do not respond to TKI treatment [54]. We initially attempted to customize and design the mass array panel aiming to achieve the specific, sensitive, and simultaneous detection of common *BCR::ABL1* mutations. In addition to resource limitations and the desire to cover all clinically relevant and hotspot mutations of *BCR::ABL1* TDK, we prioritized selecting the majority of samples that harbored multiple *BCR::ABL1* TDK mutation patterns as samples in the validation process. Finally, our mass array panel prototype simultaneously analyzed 20 hotspot mutations of *BCR::ABL1* TKD by using 18 different primer sets. These mutations were recognized as key mutational markers of resistance to several available TKIs, including imatinib, dasatinib, nilotinib, busotinib, and ponatinib [59–61].

Although mass array technology has several advantages for genotyping analysis in many applications, the development of target-specific mass array panels for mutational analysis of *BCR::ABL1* TKD in CML patients undergoing TKI treatment has remained limited in some laboratories. One explanation for this is that the cost of mass spectrometry is prohibitive, so it is not universally available. Laboratories should establish their own database of *BCR::ABL1* TKD mutations, which could be used for developing customized panels of desired targets for detecting mutations spanning the sequence of interest present in the database. Furthermore, the laboratory should consider whether the ultimate goal of the developed assay is screening or detecting low-level *BCR::ABL1* TKD mutations or detecting measurable residual disease (MRD). Parker et al. developed a highly sensitive multiplexed mass array assay with LOD ranging from 0.05% to 0.50% to simultaneously detect 31 mutations of *BCR::ABL1* TKD in patients treated with nilotinib or dasatinib after they developed resistance to imatinib [62]. Additionally, this group extended their custom panel to analyze the impact of multiple

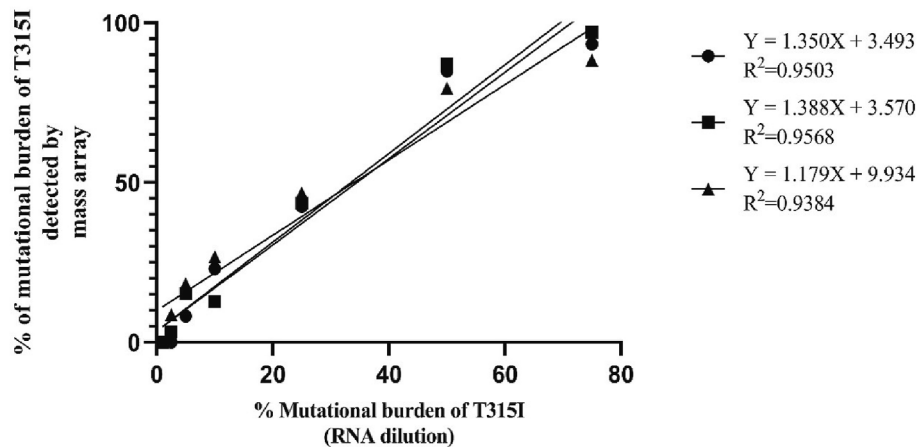


Fig. 4. A simple linear regression analysis demonstrated remarkable linearity of the dilution of T315I mutational burden, with measurable values of a mutant allele by mass array having R<sup>2</sup> values of 0.9680, 0.9503, and 0.9384.

Table 5

Operational characteristics of the mass array and other comparable methods to detect *BCR::ABL1* mutations and recurrent genetic mutations in MPNs.

Operational characteristics	Mass array	Routine laboratory assays			
		Direct sequencing for <i>BCR::ABL1</i> TKD	AS-PCR for <i>JAK2</i> V617F	CE-PCR for <i>CALR</i>	MLPA for <i>JAK2</i> , <i>CALR</i> , <i>MPL</i> , and c-Kit
Number of samples per run	1–96	1	1–10	1–4	1–4
Targets to detect per run	23	Whole of <i>ABL1</i> TKD	1	Exon 9	8
Turnaround time	1–2 days	2–3 days	1 day	1 day	2–3 days
*Cost per sample	~£28	£175	£51	£65	£121
**Overall operational complexity	Intermediate	High	Low	Intermediate	Intermediate

\*Cost per sample is related to our laboratory’s test/unit (sample).

\*\*Here, the overall operational complexity is defined as follows: high, required complicated and multiple analytical steps and instruments; intermediate, comprised multiple analytical steps but with a user-friendly analytical protocol and fewer instruments; low, required fewer analysis steps, involved user-friendly analytical tools, and was automated.

(now known as compound/polyclonal mutations) low-level *BCR::ABL1* TKD mutations on the response to ponatinib treatment. They found that the number of mutations detected by the mass array after the development of TKI resistance could predict the response to ponatinib treatment and could be used to improve the therapeutic method [63]. Similar to our data, the mass array showed advantages over direct sequencing in detecting multiple *BCR::ABL1* mutations in samples collected at similar time points. This further highlights the benefits of using mass array for the molecular monitoring of *BCR::ABL1* mutations during TKI therapy.

With regard to the performance of the mass array compared to available laboratory assays routinely used to detect *BCR::ABL1* mutations, we found that the mass array exhibited several advantages over other comparable techniques. While the mass array exhibited similar specificity to other comparable methods when analyzing *BCR::ABL1* mutations, the assay had higher sensitivity and could detect more mutations in a sample. In contrast to previous studies using plasmids containing point mutations as a standard for sensitivity study [62], we used serial proportions of RNA 100% positive for T315I mutation by direct sequencing in normal RNA isolated from peripheral blood. Hence, the LOD of our developed mass array panel is higher than those in previous studies, which could be improved given the effect of dilution of the tested RNA during cDNA synthesis and PCR reactions. We believe that our data closely reflect the dynamics of the mutational burden of a particular mutation clone during disease progression or treatment. Similar to other reports, the conventional direct sequencing method can detect the whole sequence of *BCR::ABL1* TKD. Nevertheless, the assay has several disadvantages, including high cost per sample, laboriousness, long turnaround time, and an inability to discriminate compound *BCR::ABL1* mutations (mutations occurring on the same allele) [64].

Although the mass array could detect more selected *BCR::ABL1* TKD mutations in our samples compared to the standard direct sequencing, discrepancies in results between the two techniques were observed. We found that 43% (12outof28) of the samples showed inconsistent results between mass array and direct sequencing to detect *BCR::ABL1* TKD mutations. While concordance results between the two assays for the detection of clinically significant mutations, including T315I, were observed, the mass array had superior sensitivity to direct sequencing for the analysis of L248V with del248-274, E255K, E255V, F317L, and F359C. Remarkably, the mass array could detect F317L in the same samples that were negative by direct sequencing (CML002 and CML007). Additionally, a similar results trend for the analysis of L248V with del248-274 (CML011 and CML013), E255K (CML009, CML018, and CML022), E255V (CML010, CML026, CML027), and F359C (CML009) was observed. In contrast, the mass array could not detect E279K (CML004), Y253H (CML009), F317I and F317L (CML011), and F311I (CML013), which were positive by direct sequencing. The disagreement in data might have occurred due to limitations of the individual compared methods. Currently, standard direct sequencing is generally used to screen mutations in the *BCR::ABL1* TKD and confirm the identified mutations. However, the limitation of the assay is its comparatively low sensitivity compared to mass array and other molecular methods. Like other molecular genetic assays designed for hot-spot mutation analysis, the mass array could not detect novel mutations or mutations not included in the designed panel. Therefore, we could not detect the F311I mutation in this report. The quality and amount of starting RNA or DNA could directly affect the results of several genotypic assays, including the mass array [65]. Moreover, the stability and efficacy of chemical components, especially in PCR operation, are

reflected in the quality of mass array data. Furthermore, the assay with multiple hands-on steps during PCR reduces reproducibility and increases the chance of amplicon contamination [66]. Hence, duplicating mass array and subsequently using other genotyping methods such as AS-PCR, digital PCR, and next generation sequencing are highly recommended to avoid false negative and false positive results by the mass array.

The prevalence of the selected targets of *BCR::ABL1* TKD mutations in our customized mass array panel varies mainly depending on the studies' cohorts and the *BCR::ABL1* TKD detection methods. In our previous report using the direct sequencing technique, we observed that T315I is the most frequent *BCR::ABL1* TKD mutation distributed in CML patients (28/245, 11%). Similar to other studies, T315I was reported to be the most prevalent TKD mutation among CML patients during TKI therapy [47,54,67]. Notably, T315I is recognized as the "gatekeeper" mutation that contributes to resistance to all currently available TKIs except ponatinib, asciminib, and olverembatinib (HQP1351) [68–69]. Therefore, assessing T315I mutation in CML patients who exhibit signs of not responding to TKIs is crucial for managing CML during therapy. Consequently, in this report, we prioritized T315I mutation to study the assay performances of our customized mass array panel. Besides, T315I is one of the mutations spanning *BCR::ABL1* TKD that we could observe as a homozygous mutation pattern by direct sequencing. However, it is also important to further analyze the assay performances of the established mass array panel by using other selected targets of *BCR::ABL1* TKD mutations and common mutations in MPN for improvement. Other *BCR::ABL1* TKD mutations selected for our mass array panel have been reported in various studies with different observed frequencies and the degree of response to TKI treatment. In our database, the prevalences of clinically significant *BCR::ABL1* TKD mutations recommended by the ELN guideline for adjusting the use of available TKIs were following 2% of E255K, 1% of F317L, 1% of F359V, 0.4% of Y253H, and none of T315A, V299L, respectively [54]. Our data was comparable to other studies using standard direct sequencing to detect *BCR::ABL1* TKD mutations [14,46–47,67]. Although we could not identify the rare L248V and its alternative splicing 248-274del mutations during the establishment of our initial *BCR::ABL1* TKD mutation database, currently, we can detect one patient with L248V/248-274del/T315I (patient CML006). Since the L248V/248-274del mutation has been reported to be associated with TKIs resistance [70–72], we have included this mutation in our mass array panel.

Mutations spanning the P-loop of *BCR::ABL1*, including G250E and Q252H, have been reported to be associated with decreased response to TKIs [69–70,73]. In our previous report, the Q252H mutation was observed at a lower frequency (0.4%) compared to other studies, ranging from 3 to 5% [14,46,74]. Additionally, the G250E mutation was identified as part of multiple mutation patterns. Remarkably, G250E was concurrently identified with clinically significant Y253H and T315I mutations [54]. There is evidence indicating that multiple mutations involving the P-loop and T315I mutations contribute to resistance to multiple available TKIs [44,75]. The standard direct sequencing method has low sensitivity and cannot distinguish the subpopulation of *BCR::ABL1* mutant clones. Thus, more precise and highly sensitive assays for analysis of *BCR::ABL1* TKD mutations are very important to identify low-level mutations with potential clinical significance at the time of diagnosis and during treatment to optimize the use of TKIs.

M351T and F359I have been reported to be involved in TKI resistance in CML [59]. Patients with the M351T mutation were associated with poor prognosis in imatinib-treated CML compared with those without the mutation [67]. This mutation has been observed to be relatively common in several studies [14,47,73]. However, we could not detect this mutation in samples deposited in our CML database. The F359 mutation, including F359V, F359I, and F359C, is also recognized as a critical *BCR::ABL1* TKD mutation that confers resistance to imatinib treatment. Identifying the F359 mutation could benefit patients with imatinib resistance by switching to secondary TKI, dasatinib [76]. F359

was identified at a low frequency (1%) in our previous study, which is comparable to other reports [14,46–47,54,67]. While optimizing our mass array panel, we could successfully detect F359C and F359V mutations. Nevertheless, our pre-designed mass array panel could not analyze the F359I mutation. Hence, we did not include M351T and F359I mutations in our pre-designed mass array panel.

During the past decade, alongside the improvement of the management of CML, several molecular techniques have been applied and introduced for identifying and monitoring *BCR::ABL1* mutations during TKI therapy. The performance of currently available methods is listed and discussed in Table 6. Recently, evidence has emerged of the use of high-throughput, next-generation sequencing (NGS) to identify and monitor *BCR::ABL1* mutations in CML patients. NGS has several advantages over other molecular genetic techniques, such as in terms of the number of mutations detected, assay throughput, automation, cost, and the ability to provide both qualitative and quantitative analyses. More importantly, the assay enables the rapid identification, quantification, and detection of multiple *BCR::ABL1* TKD mutation patterns in a sample. A panel of Italian clinicians and biologists suggested the indications for NGS testing for detecting *BCR::ABL1* mutations while managing patients with TKI treatment [59]. Moreover, the application of NGS has been widely used for the analysis of clonal diversity, especially CML clones with TKI resistance, and extensively used for monitoring the level of *BCR::ABL1* TKD mutations during therapy [49,52,59,77–79]. A recent multicenter study (NEXT-in-CML) found that NGS could provide a clearer picture of *BCR::ABL1* mutation status in CML patients with failure or warning responses to TKI therapy. Additionally, they observed that CML clones with TKI resistance harboring low-level mutations experience selective expansion if the TKI is not changed or an inappropriate TKI or TKI dose is chosen [51]. Apart from the potential use of NGS for *BCR::ABL1* mutation analysis, recent reports have demonstrated the applications of NGS for identifying other genetic alterations involved in establishing TKI resistance, such as ASXL1, RUNX, and IKZF1 mutations [29,80–83]. However, the use of NGS is still limited to particular laboratory settings, and there is no well-established standard for assay operation and data interpretation.

In this study, we improved our pre-designed mass array panel by adding genetic markers, including *JAK2* V617F, calreticulin (*CALR*) types 1 and 2, and *MPL* W515K/L, which have been used for the classification of classical MPNs (PV, ET, and PMF). Although several key performance indexes such as LOD and reproducibility of the assay in detecting the mutations were not investigated in this study, we demonstrated that our modified mass array panel is specific for detecting mutations common to MPNs. Additionally, the results were concordant with comparable assays used in our laboratory. Similar to the limitation of the mass array for analyzing *BCR::ABL1* TKD mutations, this customized panel could not detect mutations apart from *JAK2* V617F, calreticulin (*CALR*) types 1 and 2, and *MPL* W515K/L. Recently, evidence has emerged indicating that the mass array can be applied for identifying *JAK2* and *MPL* hotspot mutations in MPNs. Zhan et al. demonstrated that the mass array produced data consistent with those of AS-PCR for detecting *JAK2* V617F and *MPL* W515K/L in MPNs [84]. However, less evidence indicates the possibility of applying mass array to analyze *CALR* mutations in classical MPNs. This could be because the mutation patterns of *CALR* are very heterogeneous, and some mutations involve a large genetic insertion/deletion in *CALR* exon 9 [85]. It is thus difficult to design primers for use in the PCR step of the mass array that cover all pathogenic mutations in the *CALR* coding sequence. At present, many laboratories prefer to use next-generation sequencing (NGS) to identify and monitor *JAK2* V617F, *MPL* W515K/L, and *CALR* mutations, the triple marker in myeloproliferative neoplasms, together with identifying several key driver mutations in blood cancers.

## Conclusion

In this study, we demonstrated the development of a mass array



**Table 6**  
Comparison of currently available methods for analyzing *BCR::ABL1* mutations.

Method	<i>BCR::ABL1</i> TKD target	Sensitivity	Advantage	Disadvantage	References
Direct sequencing	Whole of <i>BCR::ABL1</i> TKD	10%–25%	- Mutation identification - Semi-quantitative	- Low sensitivity - Cannot distinct compound/polyclonal mutations - High cost per sample - Labor-intensive - Long TAT	[37,60,64]
DHPLC	Whole of <i>BCR::ABL1</i> TKD	1%	- Screening test - High throughput - Reasonable cost	- Needs other downstream confirmatory assays - Requires normal DNA control	[64,67,86–89]
Pyrosequencing	Hotspot mutation	5%	- High sensitivity and specificity - Quantitative assay - Not too expensive	- Not suitable for screening test (requiring mutation data) - Labor-intensive - Short amplicon length of detection	[39,90–93]
AS-PCR	Hotspot mutation	0.01%–0.001%	- Easy to perform - High sensitivity and specificity - Quantitative assay	- Not suitable for screening test (requires mutation data) - False-positive and false-negative	[94–96]
Digital PCR (dPCR)	Hotspot mutation	0.01%–0.05%	- High specificity and sensitivity - Quantitative - Short turnaround time - Could be multiplexed - Not too expensive	- Not suitable for screening test (requiring mutation data)	[97–98]
Mass array	Hotspot mutation	0.05%–2.5%	- High specificity and sensitivity - Screening of hotspot <i>BCR::ABL1</i> TKD mutations - Quantitative - Short TAT - Not too expensive	- Cannot detect novel mutations or additional variants - Cannot distinguish compound/polyclonal mutations	This report, [62,99]
NGS	Whole of <i>BCR::ABL1</i> TKD	1%–3%	- Early identification and quantification - Able to distinguish compound/polyclonal mutations (subclonal identification)	- Expensive - Labor-intensive - Long TAT - Not well standardized and poor data implementation	[51,59]

panel with high sensitivity for the analysis of clinically important *BCR::ABL1* TKD mutations. Moreover, we improved the assay panel by adding primers specific for detecting common genetic alterations in MPNs, which have been reported to be associated with poor outcomes in the management of atypical MPNs/CML. The customized mass array was shown to be superior to our currently available assays and feasible for the screening and monitoring of hotspot mutations spanning *BCR::ABL1* TKD. Since increasing knowledge of molecular mechanisms leads to CML cells becoming resistant to TKIs and the development of new effective TKIs such as asciminib, we recommend that this assay panel be revised and improved at regular intervals, including by adding more clinically significant *BCR::ABL1* mutations and other driving mutations involved in tumorigenesis of myeloid leukemia, such as *DNMT3A* and *ASXL1*, *IDH1*, and *SETBP1* that contribute to TKIs resistance, in line with the improvement in assay performances (e.g., specificity, sensitivity, and cost).

### Ethical approval

This work was approved by the Ethics Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Thailand, and followed the principles of the Declaration of Helsinki (ID MURA2019/90).

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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