1,3-Butadiene, CML and the t(9:22) translocation: A Reality Check

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1,3-Butadiene and CML

Some epidemiological studies have linked 1,3-butadiene exposures to leukemia

Associations have been with sub-types of leukemia

CML

Lymphocytic leukemia (not further specified)*

*** To a lesser extent**

Chronic Myeloid Leukemia

CML is an instructive human malignancy in that its mutational basis has been well established

Defined genetically by the Philadelphia chromosome (Ph Chromosome)

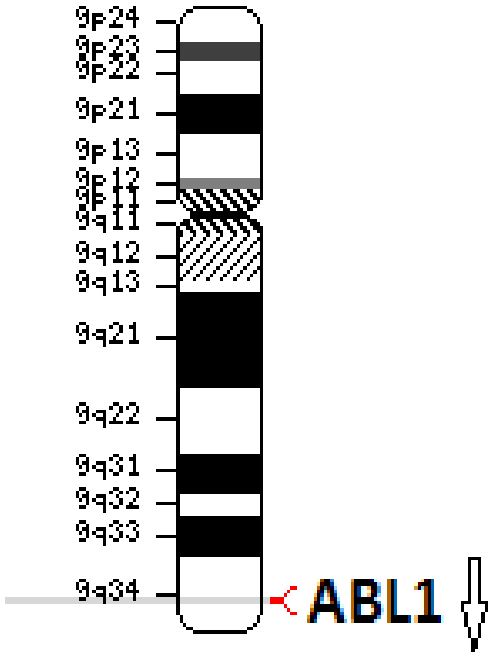
Ph chromosome is a translocation between chromosomes 9 and 22 [t(9:22)]

t(9:22) forms a fusion of proto-oncogene *ABL* (chromosome 9) with breakpoint cluster region (*BCR*) on chromosome 22.

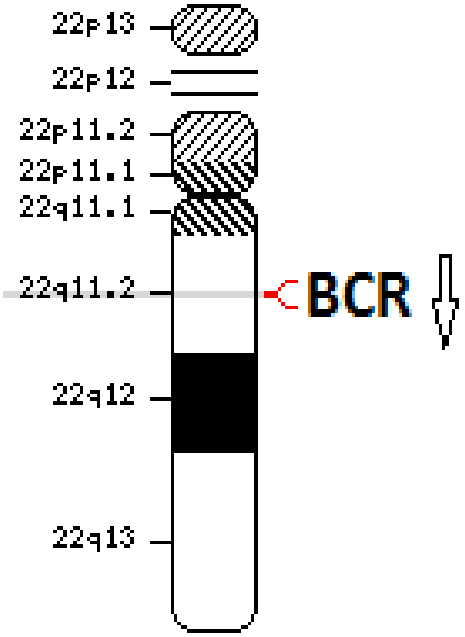
Fusion produces a hybrid tyrosine kinase that is constitutively expressed.

Hybrid tyrosine kinase is critical for development of CML as demonstrated by the clinical efficacy of inhibitors of this hybrid kinase in inducing remissions.

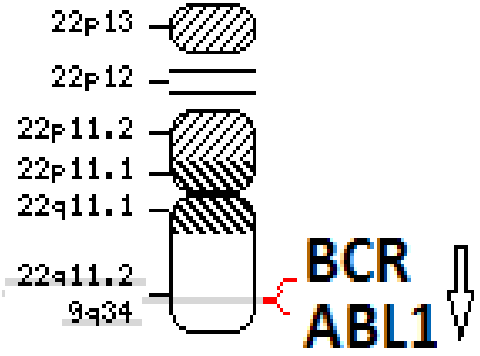
The Philadelphia chromosome; t(9:22)



chr 9



chr 22



Ph chr

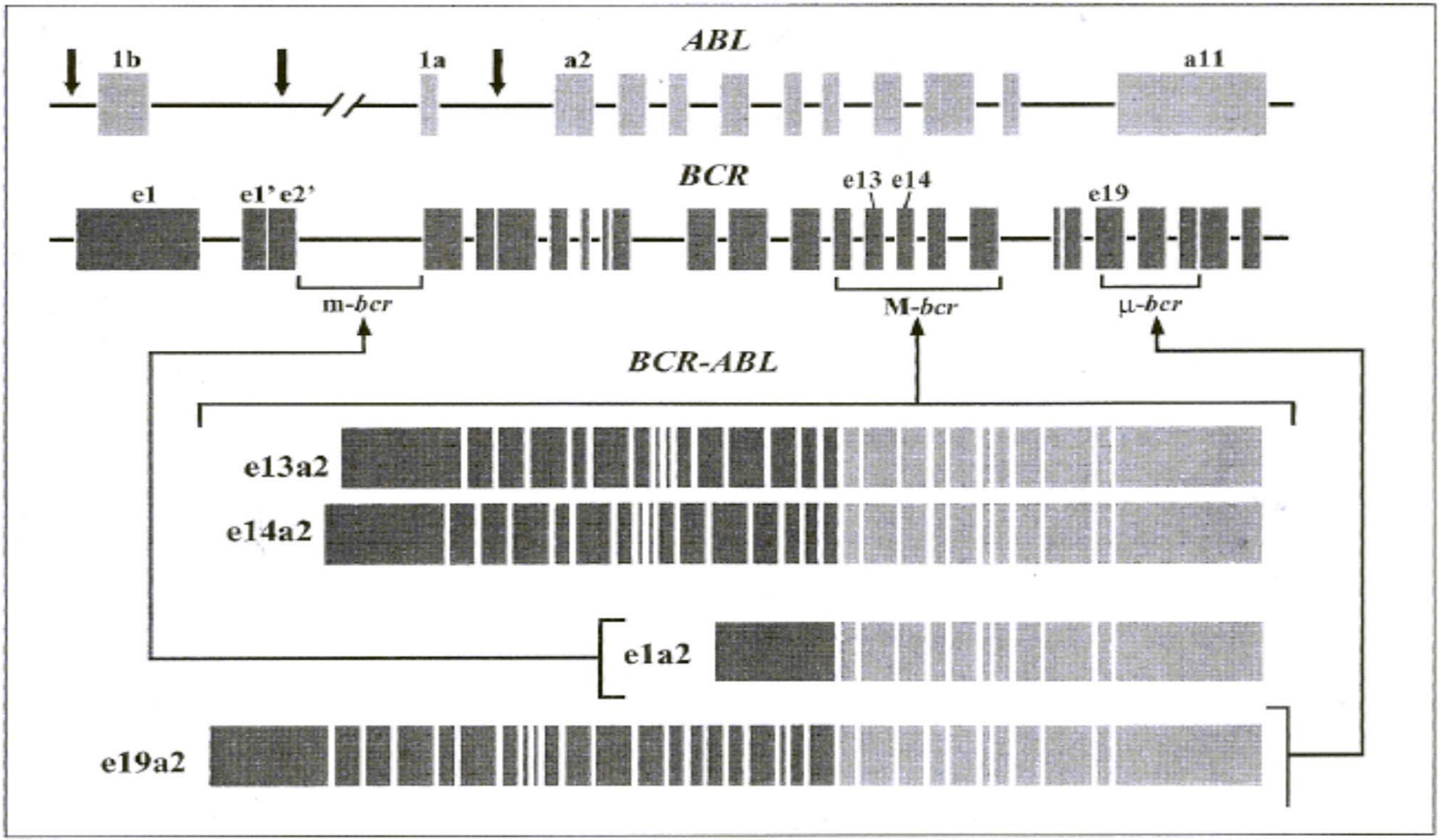


Fig. 2. Breakpoints in the *ABL* gene (indicated by arrows) and the three BCRs regions in the *BCR* gene (m-*bcr*, M-*bcr* and μ-*bcr*) (top). The fusion transcripts derived from each of the respective BCRs are also shown (bottom).

t(9:22) and CML

The only mutational event necessary for CML

Ras and other signaling pathways are aberrant in CML due to down-stream functions of the hybrid tyrosine kinase and not to mutations in these oncogenes.

Chronic Myeloid Leukemia

Strong association with ionizing radiation

Association with chemicals has been challenged

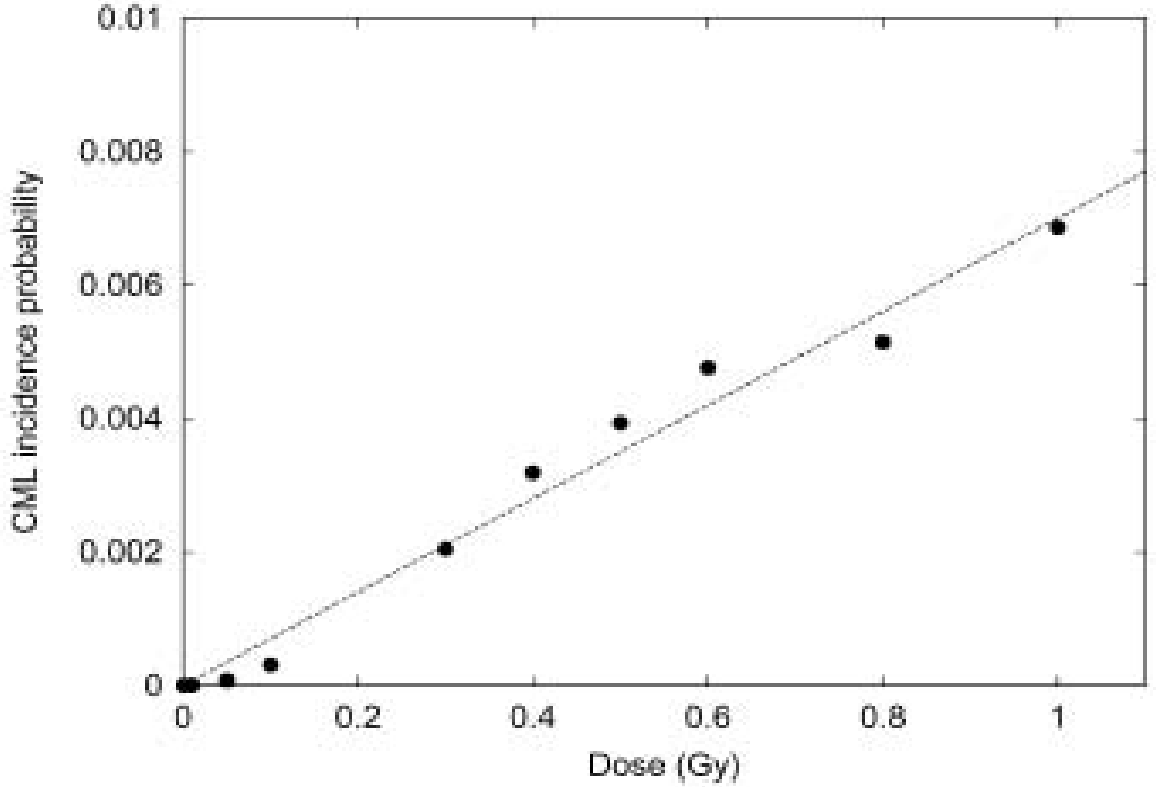


Fig. 2 Calculated dose-response for the lifetime probability of chronic myeloid leukemia (CML) induction in the dose range 0–1 Gy. *Points* represent outcomes of simulations carried out at different dose values, the *line* is a linear fit

Ballarini and Ottolenghi 2004

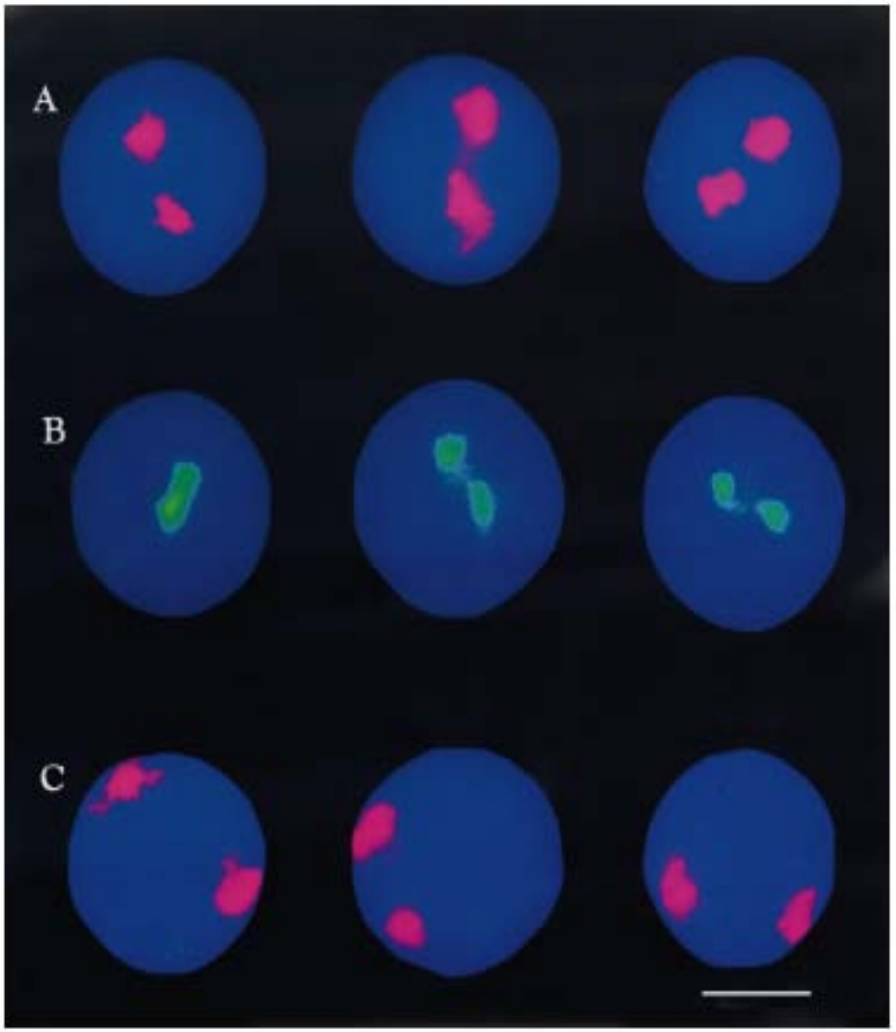


Fig. 1. Representative examples of two-dimensional (2D) images of territories of chromosomes 9 (**A**), 22 (**B**) and 8 (**C**) in non-stimulated human lymphocytes. Chromosomes 9 and 22 are located near the center of the nucleus, in contrast to chromosomes 8, which are located close to the nuclear membrane. *Bar* represents 5 μm

Kozubek *et al.* 1999

The Oncologist®

Leukemias

Is There an Entity of Chemically Induced *BCR-ABL*–Positive Chronic Myelogenous Leukemia?

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Key Words. Myelogenous leukemia • Secondary leukemia • Radiation • Chemotherapy • Benzene • Chromosomes

2008

PURPOSE OF PROPOSED RESEARCH

"Its ultimate purpose is to determine if DEB and other metabolites of BD, alone or with other agents (e.g. styrene, dimethyldithiocarbamate [DMDTC]), can produce the only mutational event, i.e. one of three specific t(9:22) chromosome translocations, that initiates the human malignancy chronic myeloid leukemia (CML)".*

*** Original application**

SPECIFIC AIMS FOR PHASE 1

Characterize cell lines, i.e. 100 % b2a2, b3a2 and e1a2 translocations in lines BV173, K562 and SD-1, respectively, and rare to no translocations in potential target lines TK6, HL60 and KG1.

Optimization real-time PCR methods.

Sequence PCR products to verify the specific translocations.

Determine the sensitivity of optimized real-time PCR method.

Induce pathogenic t(9:22) translocations in suitable target cells.

Table 1 - Cells lines used in the experiments

Cell Line	Source	t(9:22) Rearrangement	Sequence of BCR-ABL cDNA
HL-60	ATCC	None	No BCR-ABL product
KG-1	ATCC	None	No BCR-ABL product
TK-6	DSMZ	None	No BCR-ABL product
BV173	DSMZ	b2a2	BCR ex13-ABL ex2
K562	ATCC	b3a2	BCR ex13-BCR ex14-ABL ex2
Ku812	ATCC	b3a2	BCR ex13-BCR ex14-ABL ex2
Meg01	ATCC	b2a2	BCR ex13-ABL ex2
SD-1	DSMZ	e1a2	BCR ex1-ABL ex2
SubB15	ATCC	e1a2	BCR ex1-ABL ex2

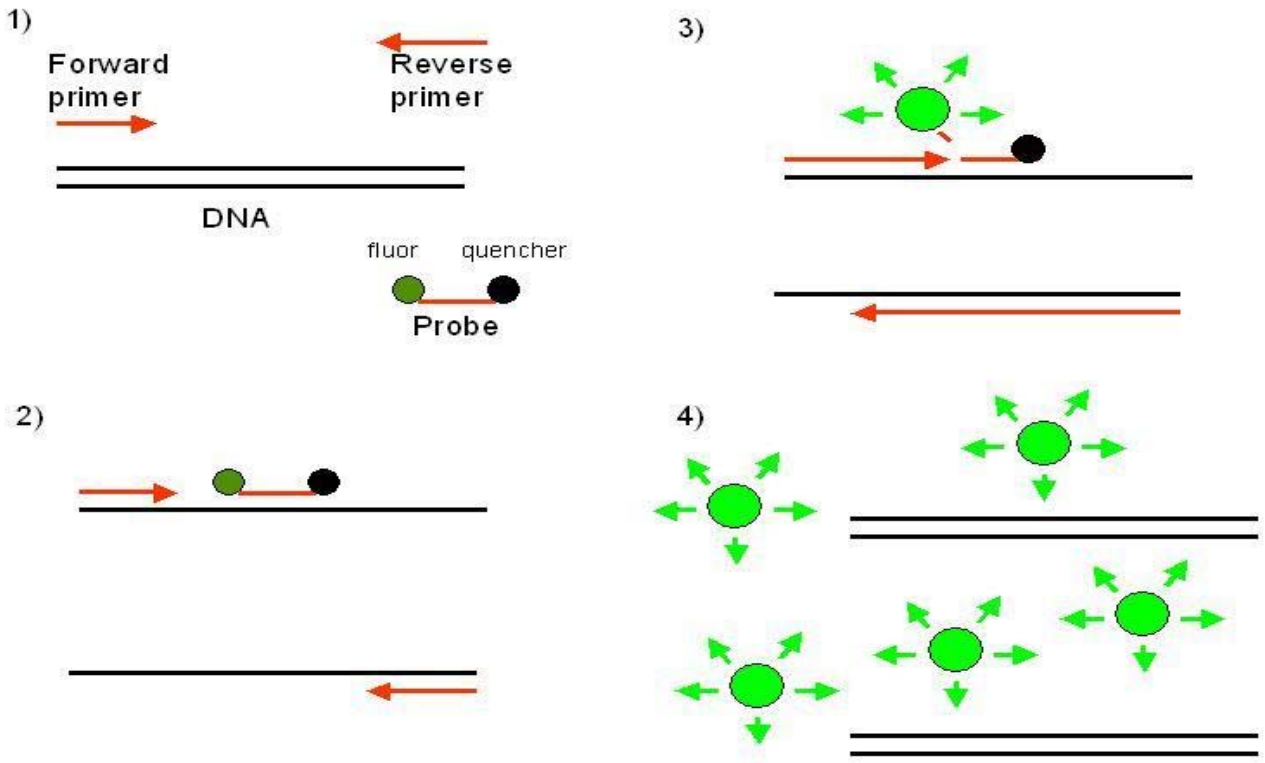
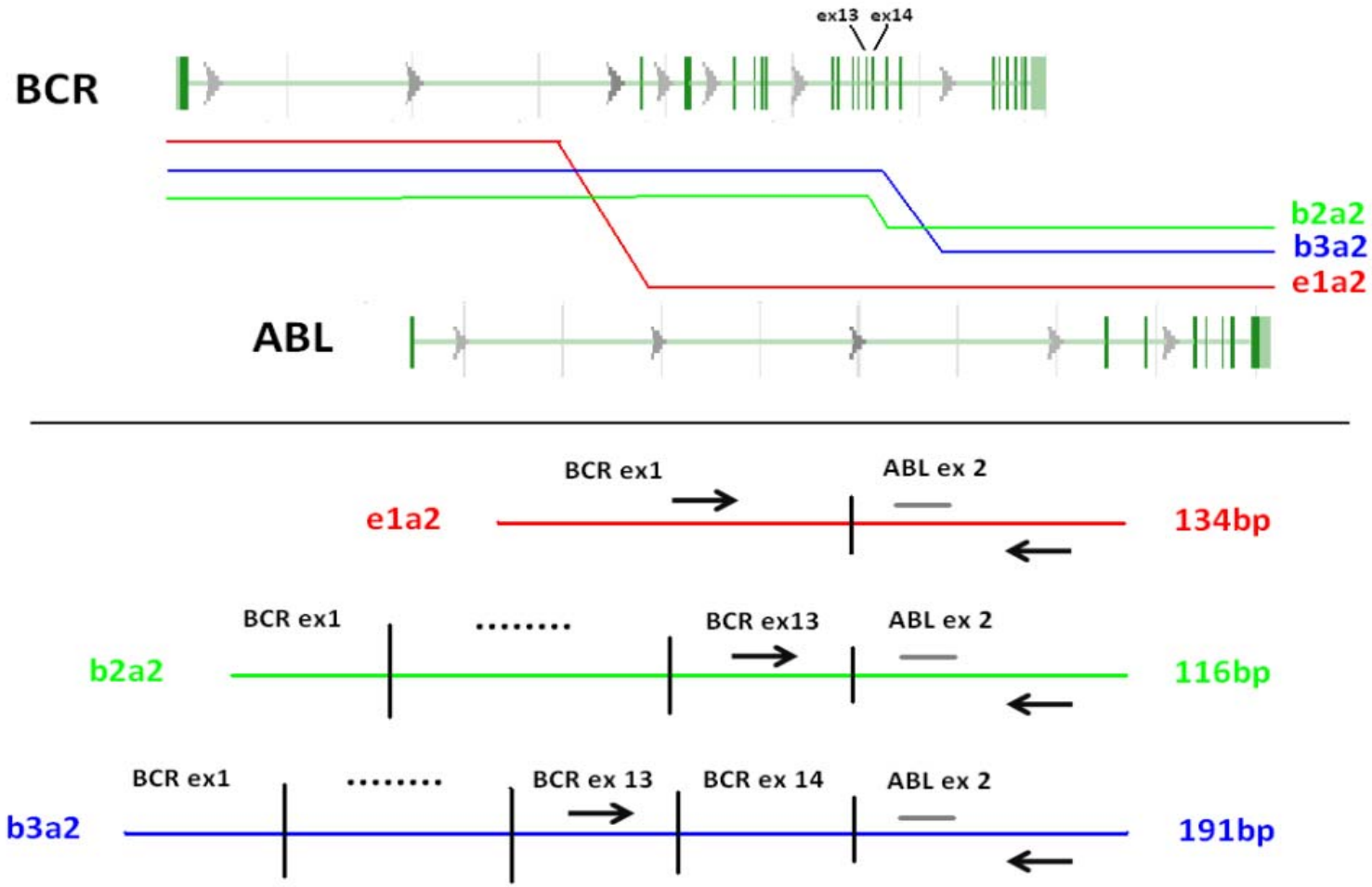
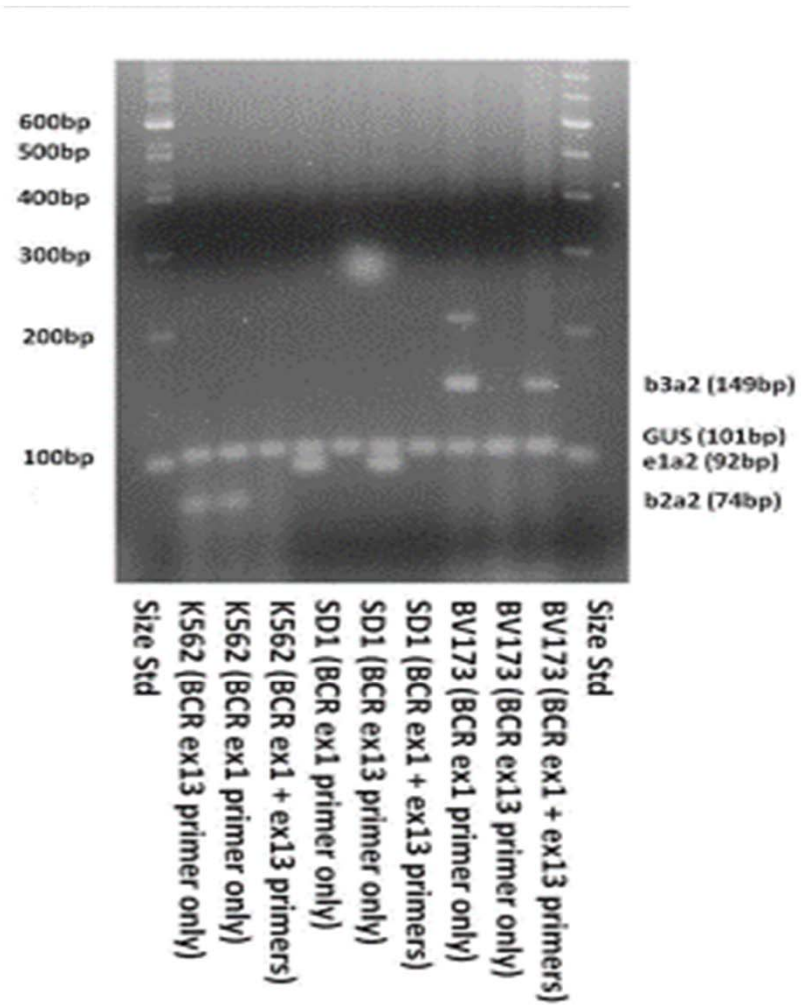


FIGURE 3X – TaqMan® assay. 1) PCR is performed with DNA (or cDNA), forward and reverse primers and the probe which has a fluorescent molecule on the 5' end (usually FAM) which is quenched by another dye (often a black hole quescher (BHQ)) on the 3' end. 2) During the annealing phase of the PCR, the primers and probe bind to the PCR product but the fluor is quenched by the quencher. 3) The probe is degraded by the polymerase as the PCR primer is elongated and as a result, the fluorophore is separated from the quencher and is free to report fluorescence. 4) During each PCR cycle, more and more free fluorophore is generated yielding fluorescence directly proportional to the cycle number and the amount of input DNA.

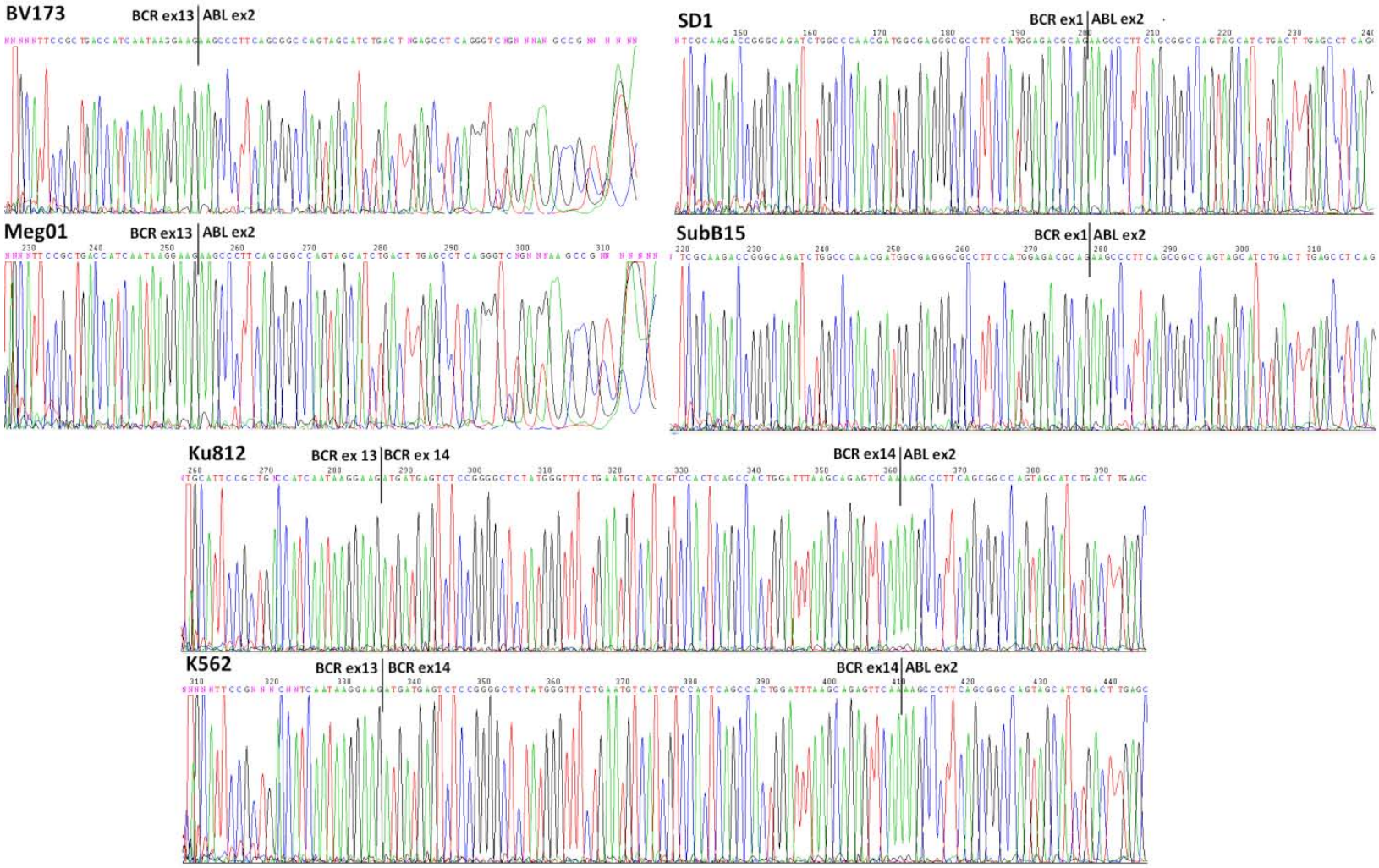
Primer placement and *BCR/ABL products*



Agarose Gel of BCR-ABL products from Cell Lines (Aim 3)



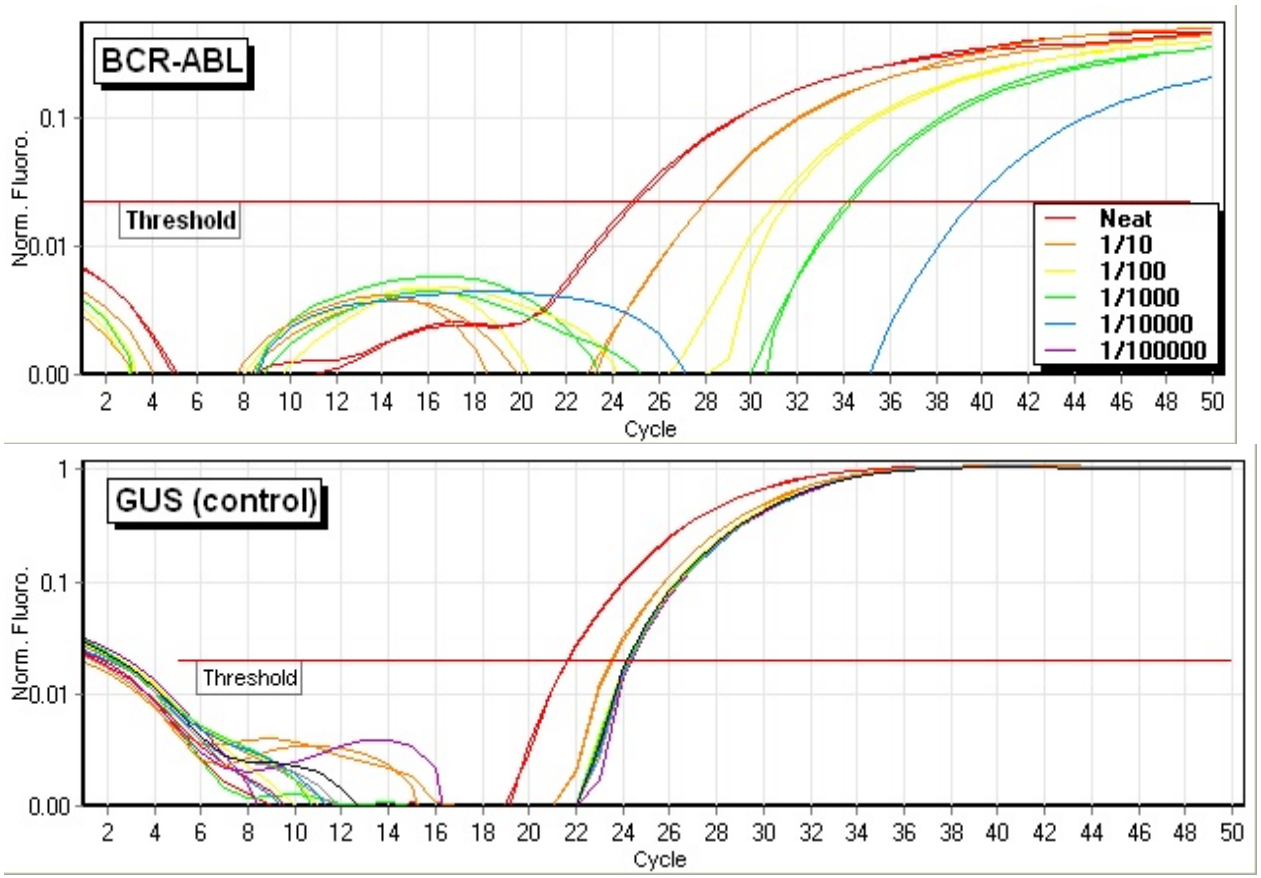
Sequences of t(9:22) translocation products amplified from each cell line



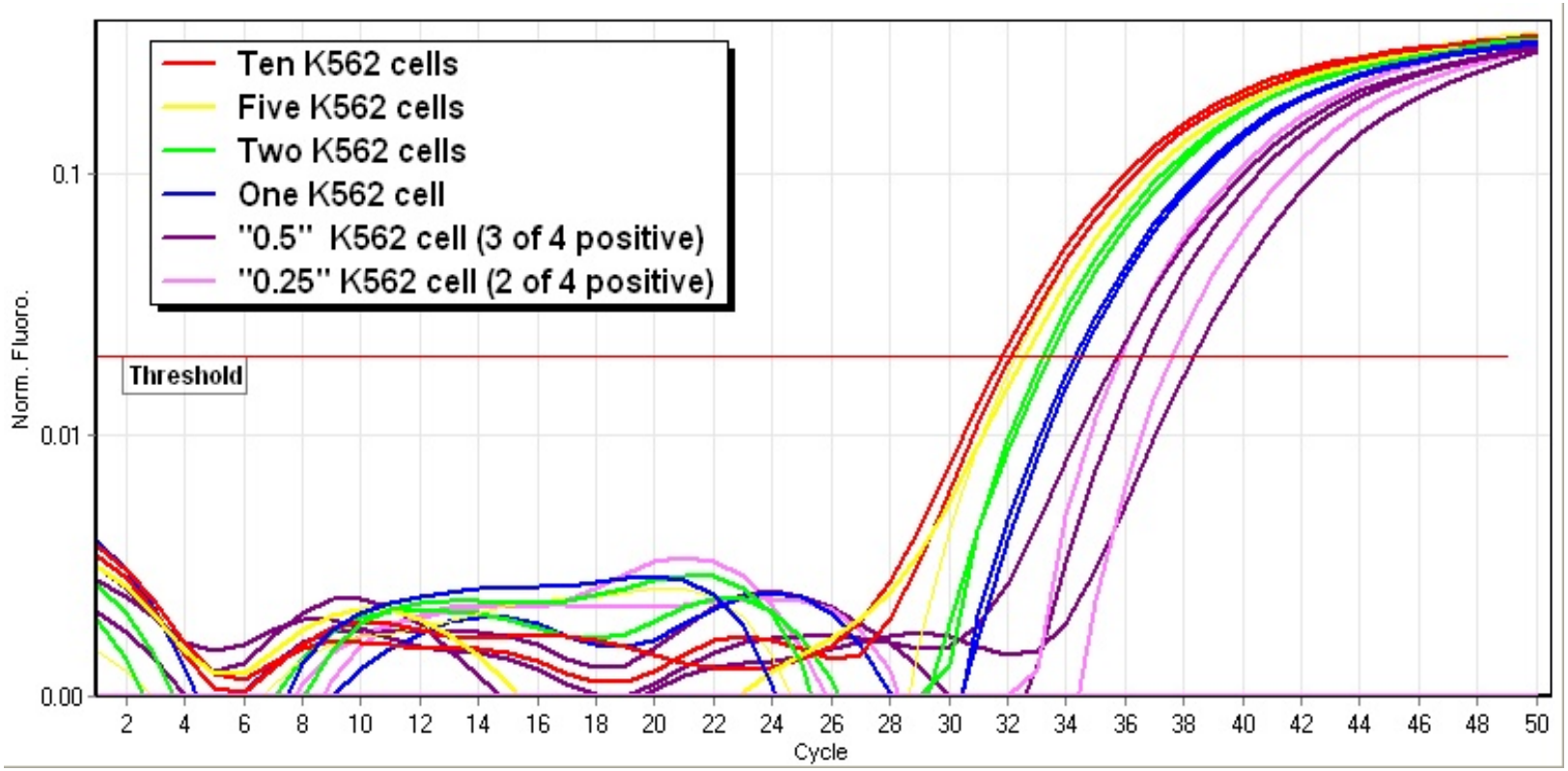
Sensitivity of Assay

A. Serial dilution of mRNA from K562 with TK6 (negative control) – neat to 10^7 (also 10^8)
B. Picking of single K562 cells to mix with 10^6 TK6 cells (1, 2, 5 and 10 cells/ 10^6 TK6)
C. Serial dilution of cells – K562, SD1 or BV173 diluted with TK6. Seven pellets were prepared containing 10^7 cells in total with serial dilutions from one mutant cell per 10 cells to one mutant cell per 10^7 cells.

Serial dilution of mRNA (K562)



Sensitivity with Individual cells

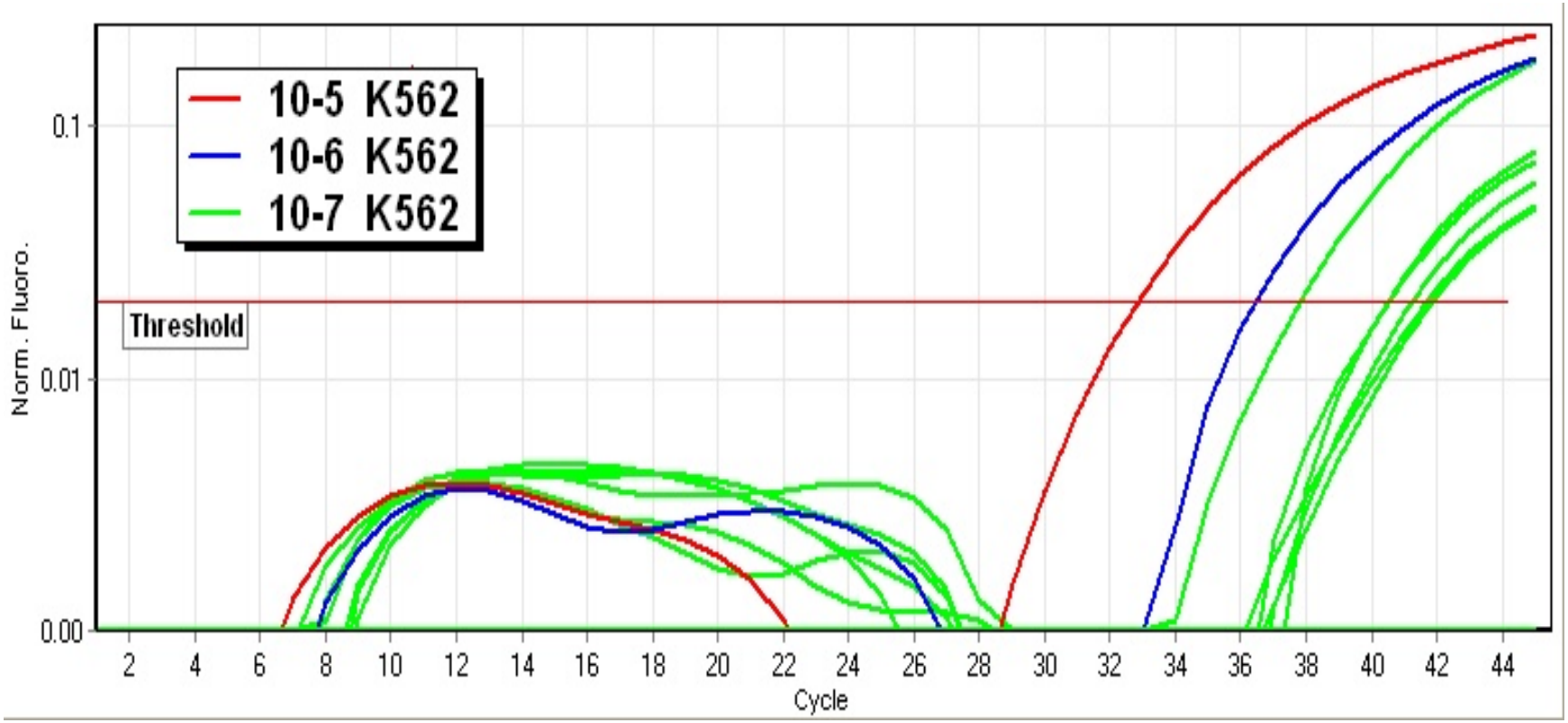


"0.5" and "0.25" cells were created by diluting the one cell mRNA extract by 1/2 and 1/4, respectively.

Serial dilution of cells

Dilution of BCR-ABL cells with TK6 (control cells)	K562 [# positive/# total tubes (% positive)]	BV173 [# positive/# total tubes (% positive)]	SD1 [# positive/# total tubes (% positive)]
10^{-5}	4/4 (100%)	8/8 (100%)	12/12 (100%)
10^{-6}	10/10 (100%)	5/14 (36%)	11/12 (92%)
10^{-7}	12/18 (67%)	1/20 (5%)	11/21 (52%)
10^{-8} (1:10 dilution of 10^{-7} mRNA)	3/8 (37%)	ND	ND

Example of serial dilution of cells (K562)



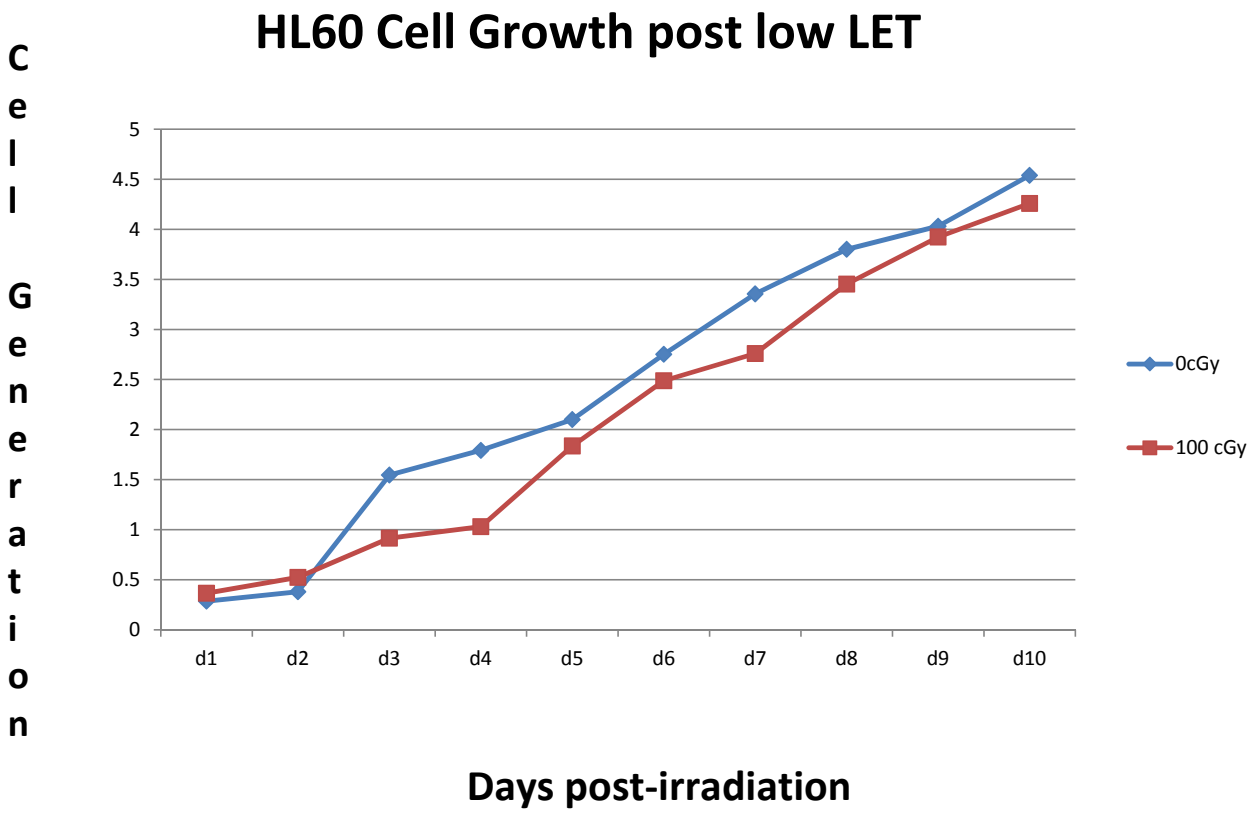
An additional test of the 10^{-7} cell mRNA diluted 1/10 ("10^{-8}") gave 3/8 positives.

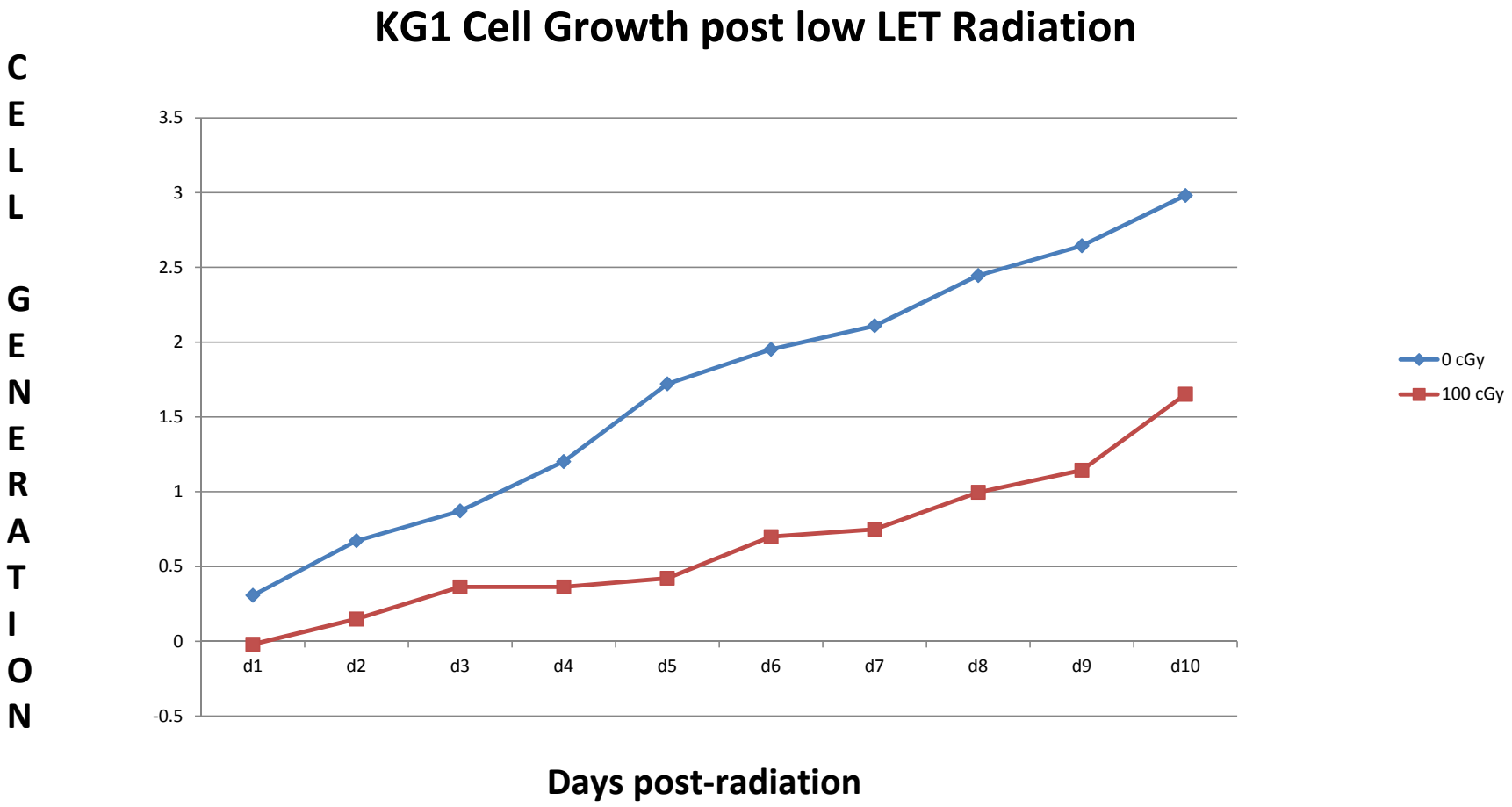
ADDITIONAL SPECIFIC AIMS

DIFFERENTIATE INDUCTION FROM RECOVERY

EXPRESS INDUCTION IN TERMS OF TRANSLOCATED CELLS RATHER THAN TRANSLOCATED cDNA MOLECULES.

Concentrate studies on HL60 cells


HL60 Cell Growth post low LET
Cell Generation
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
0cGy
100 cGy
Days post-irradiation


KG1 Cell Growth post low LET Radiation
CELL GENERATION
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
d1
d2
d3
d4
d5
d6
d7
d8
d9
d10
0 cGy
100 cGy
Days post-radiation

PROTOCOL FOR RADIATION STUDIES

Irradiate cells in mass cultures on day 0.

Determine radiation killing by decrease in cloning efficiency . Cells are plated for cloning immediately after radiation and counted on day 16.

Plate all remaining cells.

Culture *in vitro* for 24 hours for cell lines (HL60) to allow for the translocation to occur and for its genotypic and phenotypic expression (i.e. production of mRNA from the translocated DNA) but also to not allow significant cell proliferation.

Count viable cells in radiated flasks at time of termination.

Remove and pellet 40 aliquots of 4 X 10^5 or more cells each (minimum total = 1.6 X 10^7 cells) from each radiation level flask at termination.

CALCULATION OF TRANSLOCATION FREQUENCIES

Assume Poisson distribution of cells with translocations.

Consider each aliquot of cells to be a single sample of "n" cells.*

Observe frequency of aliquots without translocations = no PCR signal.

Solve for "x" from the equation for the P_0 (no signal) class of aliquots.

$P_0=e^{-x}$ where "x" is the average number of translocated cells per aliquot; x/"n" cells = translocation frequency.

*** "n" = 400,000 or 800,000 cells (i.e. 1.6 or 3.2 X 10^7 HL60 cells per experiment**

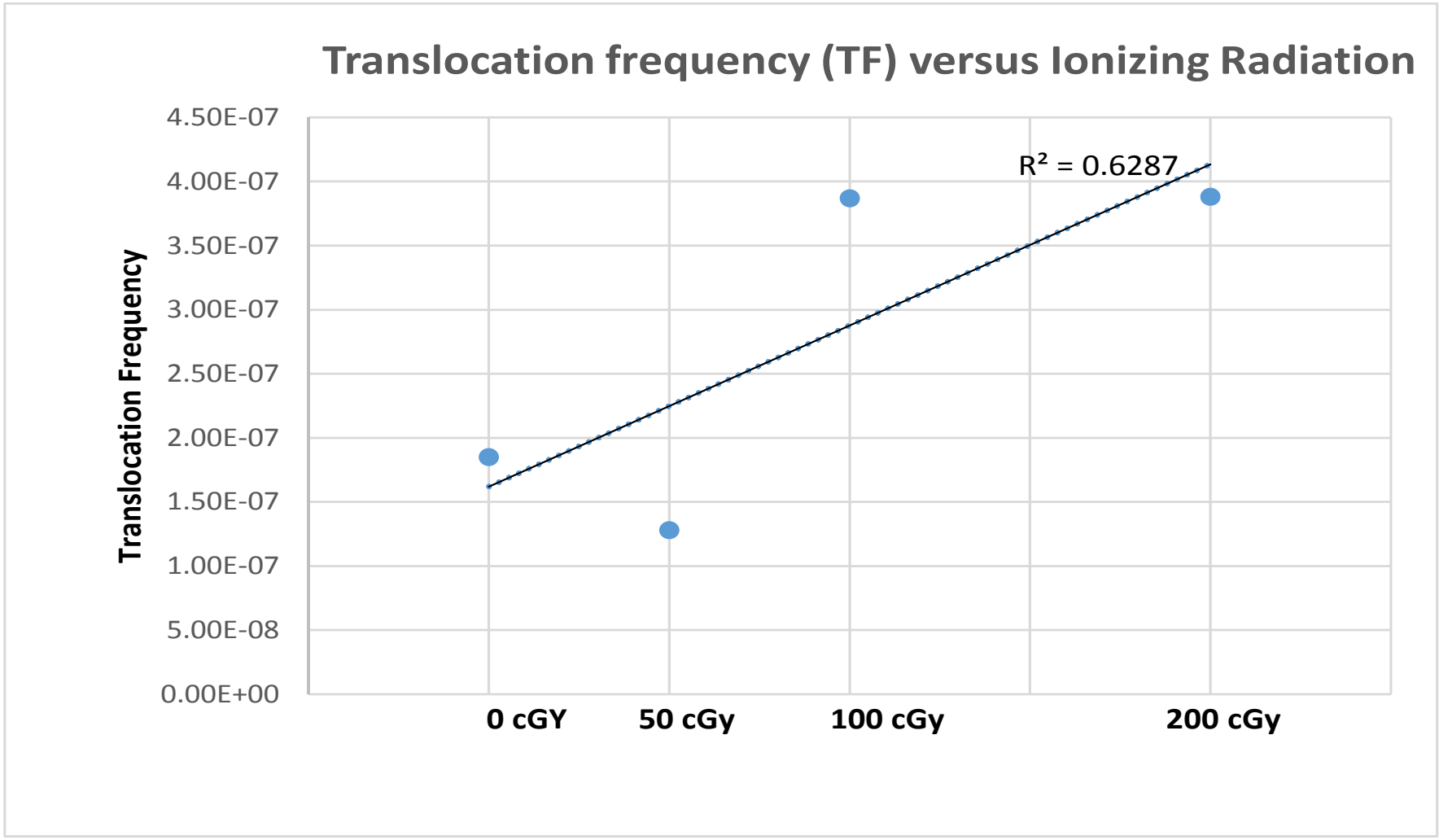
t(9:22) translocation frequencies versus radiation dose

Expt #	0 cGy	50 cGy	100 cGy	200 cGy
1	1.80E-07	ND	7.20E-07	ND
2	1.30E-07	1.90E-07	6.40E-07	4.10E-07
3	3.30E-07	1.30E-07	1.90E-07	4.10E-07
4	1.30E-07	6.30E-08	0	2.60E-07
5	1.70E-07	ND	ND	4.10E-07
6	1.70E-07	ND	ND	4.50E-07
Average	1.85E-07	1.28E-07	3.87E-07	3.88E-07

Expts. 1-4 (γ radiation, $1.6X10^{7}$ cells); Expts.5&6 (X-ray, $3.2X10^{-7}$ cells)

Confidence limits based on pooled sd = 1.733					
				Confidence limits	
		mean	se	lower	upper
	0 cGy	1.85	0.708	0.463	3.237
	50 cGy	1.28	1.009	-0.698	3.258
	100 cGy	3.87	0.875	2.155	5.585
	200 cGy	3.88	0.775	2.361	5.399

Dose-response significant (p = 0.024) when differences between experiments controlled based on regression analysis of linear model.


Translocation frequency (TF) versus Ionizing Radiation
Translocation Frequency
4.50E-07
4.00E-07
3.50E-07
3.00E-07
2.50E-07
2.00E-07
1.50E-07
1.00E-07
5.00E-08
0.00E+00
R² = 0.6287
0 cGY
50 cGy
100 cGy
200 cGy

Rearrangements seen in HL60 experiments

Expt	# of each rearrangement sequenced				
	e1a2	b2a2	b3a2	e1a2 variant	Other
0 rad					
1 (ND)					
2	4				
3	1				1-BCR ex1 only (incomplete ABLex2)
4	3		1		1-SLC17A5 (chr6) to ABLex2
5	9			2*	1-failed to sequence
6	6				
200 rad					
1	6		1V		
2	6				
3	4				
4	8				1-chr14 to ABLex2 1-SLC17A5 (chr6) to ABLex2 1-WDFY ex1 (chr2) to ABLex2
5	3			1	
6	10		1		3-SLC17A5 (chr6 to ABLex2)

*1-BCRex1 spliced to pseudo exon in ABL int 1 spliced to ABLex2, 1-deletion of 1st 3bp of ABLex2

Conclusions from studies

1. **LET ionizing radiation does induce pathogenic t(9:22) translocations.**

2. **Ionizing radiation can serve as a positive control for studies of chemical induction of t(9:22) translocations.**

3. **Assessment of the capacity of 1,3-butadiene metabolites to induce t(9:22) translocations will require acute and chronic exposures and the additional inclusion of a positive control for non-specific clastogenic effects, e.g. micronuclei.**

4. **Experiments are laborious and relatively expensive.**