Malignant Lymphomas

Variability of quantitative polymerase chain reaction detection of the *bcl-2*-IgH translocation in an international multicenter study

The t(14;18)(q32;q21) chromosomal translocation is closely associated with follicular lymphoma. Polymerase chain reaction (PCR) analysis has high sensitivity and is used to assess responses to therapy. Quantification of translocation-bearing cells is a possible advantage of real-time PCR over conventional PCR. A collaborative study comparing results from 12 international laboratories is reported.

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Polymerase chain reaction (PCR) analysis can detect malignant cells present below the level of detection of conventional staging (bone marrow morphology and computed tomography scan). However, results are complicated by both biological and technical factors. The translocation can be found in healthy individuals,[1] and in follicular lymphoma is associated with variability of breakpoints.[2] PCR is associated with false positives and false negatives, and sensitivity varies between centers. Studies suggest that reversion to PCR negative status (molecular remission), for example in follicular lymphoma after autologous bone marrow transplant, is associated with longer disease-free survival[3,4] and PCR results are reported in many clinical studies.[5] Our previous study of conventional PCR in 20 laboratories demonstrated variation in technique and a false positive rate of 28%. Sensitivity between centers differed with nested PCR being more sensitive than single round PCR.[6] Quantitative real-time PCR is considered a highly sensitive, reproducible and precise test with high specificity. This collaborative study compares techniques and results for 12 laboratories using their standard quantitative PCR methodology. Twelve laboratories were sent blood from normal donors with varying numbers of t(14;18)-bearing cells added from a cell line (RL) with a translocation in the major breakpoint region of the *bcl-2* gene.[7] Samples were sent blind and in duplicate in two rounds with differing numbers of added cells per milliliter of blood in each round. Results from the first round were made available to centers prior to despatch of the second round. Some centers were unable to take part in both rounds. All results from samples that were analyzed are presented. There were variations in methodologies for DNA extraction, PCR conditions and the number of sample replicates (Table 1). However, all centers used the same PCR machine (ABI 7700) and same enzyme. Five centers (B, D, F, J and K) used identical probes/primers but other aspects of their techniques differed. Centers provided results as number of copies of t(14;18) positive cells per milliliter of whole blood prior to being informed of the added number of translocation-positive cells. Table 2 shows the results of rounds 1 and 2. Sensitivity varied between centers; most centers detected the translocation when samples contained 100 or more *bcl-2-IgH*-positive cells per milliliter of blood. With 100 and 1000 cells added per milliliter of whole blood, 80% and 94% of the samples, respectively, were reported positive. In contrast, at levels of 20 cells/mL and 1 cell/mL, 50% and 9% of the samples were reported positive. Only one false positive was reported and this was found to have a different amplified product size indicating contamination. In comparison with the previous study of nested and single round PCR, the current results show that, for quantitative PCR, there is less variation in sensitivity with most centers detecting the translocation at 100 added cells per milliliter of whole blood and no false positives. However, a two-log variation in reported copies of the translocation persists. The current study appears to have resulted in more accurate and consistent results in the second round, suggesting improvement from feedback. This was not due to any specific recommendations about technique but may have resulted from increased attention prompted by poor results

Table 1. Methodology for *bcl-2*-IgH detection in the collaborating centers.

	Sample preparation/ extraction method	*Starting volume blood/ number PBMC*	*DNA per reaction*	*Cycles*	*Reaction volume*	*Conditions*	*Housekeeping gene*	*t(14;18) cell line in the laboratory*	*Number of sample replicates*
A	Puregene kit	1×10^7 PBMC	1μg	50	50 μL	95°C15s 61°C1min	WT K-ras	Karpas 422	10
B	Ficoll + phenol/ chloroform	10 mL	500 ng	50	50 μL	95°C30s 60°C90s	β-actin	OCI + DOHH	3
C	Ficoll + phenol ethanol ppt	10 mL	1 μg	50	50 μL	95°C15s 61°C1min	β-actin	No	3
D	Salt lysis	10 mL	500 ng	45	50 μL	95°C30s 60°C1min	Albumin	SUDHL DOHH	2
E	Boehringer DNA extraction kit	10 mL	1μg	50	50 μL	95°C15s 59°C1min	Albumin	DOHH	3
F	Salt lysis	5×10^7 PBMC	500ng	45	50 μL	95°C30s 60°C1min	Albumin	SUDHL	2
G	Lymphoprep+ alkali lysis	10 mL	1μg	50	50 μL	95°C15s 60°C1min	GAPDH	RL	3
H	Phenol/chlorform extraction	10 mL	500 ng	40	25 μL	95°C15s 60°C1min	β-2microglobulin	DOHH+RL	6-12
I	Ficoll	40 mL	400 ng	50	50 μL	95°C15s 60°C1min	GAPDH	Karpas 422+RL	5
J	Salt lysis	5×10^7 PBMC	500 ng	45	50 μL	95°C30s 60°C1min	Albumin	SUDHL	2
K	Qiagen kit	5×10^6 PBMC	500 ng	45	50 μL	95°C30s 60°C1min	Albumin	DOHH+RL	2
L	Ficoll+Puregene kit	3 mL	150 ng	45	50 μL	95°C15s 62°C1min	β-actin	OCI	7

Table 2. The numbers of copies of the translocation per milliliter of whole blood reported by each center. The numbers of added translocation-bearing cells are shown in bold.

Round 1	***0***	***0***	***1***	***1***	***30***	***30***	***100***	***100***	***1000***	***1000***
Center A	0	0	0	0	3	2	10	22	129	184
Center B	0	0	0	0	0	0	0	160	547	480
Center C	0	0	0	0	0	50	1000	20	20000	5000
Center D	0	0	0	0	0	0	0	11	160	213
Center E	0	0	0	0	10	8	6	10	81	113
Center F	0	0	0	0	0	0	7	142	36	0
Center G	0	0	0	0	240	0	0	0	2680	2080
Center H	0	0	0	0	2	3	7	3	116	68
Center I	0	0	0	0	0	0	0	0	20	20
Range	–	–	–	–	0-240	0-50	0-1000	0-160	20-20000	0-5000
Median	0	0	0	0	0	0	6	11	129	184
Mean	0	0	0	0	28	7	114	41	2641	906
Round 2	***0***	***0***	***1***	***1***	***20***	***20***	***100***	***100***	***750***	***750***
Center A	0	0	0	0	3	2	14	33	340	151
Center B	0	0	0	0	0	0	0	0	216	144
Center C	0	0	0	0	100	50	100	100	400	700
Center D	0	0	0	0	0	Broken sample	160	64	640	512
Center E	0	0	0	0	76	193	376	574	1540	3239
Center F	0	0	0	14	3	18	18	8	48	47
Center H	0	0	0	40	60	80	260	245	1200	1000
Center I	0	0	0	0	2	0	2	2	30	34
Center J	0	0	0	0	0	0	13	26	80	107
Center K	0	0	0	0	0	0	98	106	278	331
Center L	0	0	0	0	0	0	250	100	2500	6200
Range	–	–	–	0-40	0-100	0-193	0-376	0-574	30-2500	34-6200
Median	0	0	0	0	2	2	98	64	340	331
Mean	0	0	0	5	22	31	117	114	661	1133

in the first round. While the exact number of copies may be difficult to quantify reproducibly, we confirm here a close correlation between the number of added cells and the number of detected cells, at least for 100 or more copies of the gene per milliliter of blood. This factor, and the elimination of false positives, are clear advantages and make quantitative PCR potentially more useful in the clinical setting than conventional PCR.

Standardization of methodologies and re-testing in a prospective study is recommended in order to enable comparison of results from different clinical studies. By reaching a consensus of methods and validating these, it would be possible to test protocols for patients at high risk of recurrence in prospective clinical trials, assessing whether treating the PCR result will influence the course of illness.

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