Evaluation of T Cell Receptor Testing in Lymphoid Neoplasms

Results of a Multicenter Study of 29 Extracted DNA and Paraffin-Embedded Samples

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To evaluate current diagnostic methods used for the evaluation of T cell receptor (TCR) gene rearrangements, 24 different laboratories analyzed 29 lymphoid neoplasm samples of extracted DNA and paraffin-embedded tissue and were asked to complete a technical questionnaire related to the testing. Participating laboratories performed Southern blot and polymerase chain reaction (PCR) testing for rearrangements of the *TCRβ* chain gene and PCR for the *TCRγ* chain gene rearrangements. Of 14 laboratories performing *TCRβ* Southern blot analysis, there was complete agreement in 10 of 14 cases, with some false negative results obtained in 4 cases. No false positive results were obtained by Southern blot analysis. *TCRβ* PCR analysis was only performed by two laboratories, and only 47.1% of positive samples were detected. Twenty-one laboratory results were obtained for *TCRγ* PCR. This method showed an overall detection rate of 77.9% for T cell gene rearrangements with a 4.1% false positive rate, as compared to both *TCRγ* Southern blot analysis results and immunophenotyping. The detection rate for *TCRγ* PCR, however, significantly differed when extracted DNA samples from frozen tissue were compared to paraffin-embedded tissue (85.4% versus 65.9%; $P = 0.0005$). Significant differences in true positive results were obtained when laboratories using primers directed against multiple *TCRγ* variable regions (V1–8 plus one to three other primer sets) were compared to laboratories that used only a single set of TCR primers directed against the V1–8 ($P < 0.0001$). Other technical factors significantly affecting results were also identified. These findings provide useful data on the current state of diagnostic TCR testing, highlight the risk of

false negative results for TCR testing directed against only portions of the *TCRγ* gene, and identify limitations of testing of paraffin-embedded tissues in some laboratories. (*J Mol Diag* 2001, 3:133–140)

Lymphoid neoplasms are usually evaluated by a combination of methods that include hematoxylin and eosin stained tissue section morphology, often supplemented by immunophenotyping studies and possibly molecular genetic evaluation.^{1,2} The detection of a monoclonal cell population is usually helpful when a suspicion of malignancy exists and immunophenotyping methods for the detection of immunoglobulin light chain restriction are usually adequate for mature B cell neoplasms. No such simple immunophenotypic markers for clonality exist for T cell neoplasms, and molecular genetic testing for clonal rearrangements of T cell receptor genes are often useful in the evaluation of specimens suspicious for T cell lymphoma or leukemia.

The four T cell receptor genes undergo variable-diversity-joining (VDJ) region or variable-joining (VJ) region rearrangements as part of normal T cell development, similar to the immunoglobulin heavy chain and light chain gene rearrangements of B cell development.^{3,4} The T cell receptor δ locus (*TCRδ*) at chromosome region 14q11 is the first to rearrange, followed by *TCRγ* at 7q15, *TCRβ* at 7q34, and, finally, *TCRα* at 14q11. Approximately 95% of circulating T cells undergo all four rearrangements, but a small percentage of cells (γ/δ T cells) only undergo rearrangement of the first two genes. In the past, the most commonly used methods of detecting T cell receptor gene rearrangements used Southern blot analysis directed against the constant or joining regions of the *TCRβ* gene.⁵ Southern blot methods, however, are time-consuming, labor intensive, require a relatively large amount of fresh or frozen tissue, and require at least 5 to 10% clonal cells in the sample for detection. More recently,

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polymerase chain reaction (PCR)-based methods of detection of gene rearrangements have been used.² These methods target rearrangements of either the *TCR β* gene or the *TCR γ* gene; but because the *TCR γ* variable region is less complex than the *TCR β* variable region, many laboratories prefer *TCR γ* testing. These methods are more rapid, require smaller amounts of tissue, may be used in paraffin-embedded tissues and can detect a smaller percentage of clonal cells than Southern blot analysis.

To evaluate the utility and methodology used in laboratories performing T cell receptor clonality studies, the authors circulated a total of 29 samples of paraffin-embedded tissue and DNA extracted from frozen tissue from B and T cell lymphomas or leukemias to 21 diagnostic laboratories for PCR testing. Thirteen laboratories also performed Southern blot analysis on 14 of the samples, resulting in a total of 24 participating laboratories. The results of this sample exchange provide more information about the different testing methodologies used in diagnostic laboratories and their use in fresh/frozen and paraffin-embedded samples.

Materials and Methods

Members of the Association for Molecular Pathology were surveyed for interest in participating in the sample exchange. Thirty respondents were sent samples and questionnaires, and responses were received from 24 laboratories. Participants were generally diagnostic laboratories and they were asked to perform their routine diagnostic assays.

Frozen cells and available corresponding paraffin-embedded tissue of archived lymphoma and leukemia samples originally diagnosed at the Oregon Health Sciences University Department of Pathology were retrieved for use in this study. Lineage was assigned based on prior immunophenotyping studies in conjunction with morphological evaluation. Cases were classified according to the Revised European-American Classification of lymphoid neoplasms and the proposed World Health Organization Classification.^{1,6} Extracted DNA from 16 lymphoma/leukemia samples (9 B-lineage: 7 follicular lymphoma, 2 chronic lymphocytic leukemia/small lymphocytic lymphoma; 7 T-lineage: 4 peripheral T cell lymphoma, 1 mycosis fungoides/Sézary syndrome, 2 lymphoblastic lymphoma) was aliquoted and distributed to each participating laboratory. For DNA extraction, frozen cells were thawed, pelleted, and resuspended in an equal volume of lysis buffer, consisting of 50M Tris-HCL, pH 8.0, 100 mmol/L EDTA, supplemented with 0.9 mg/ml proteinase K and 0.5% SDS. The lysis mix was incubated for 16 hours at 55°C and extracted twice with an equal volume of phenol and once with chloroform/isoamylalcohol at a 24:1 ratio. DNA was precipitated with 0.1 volume of 7.5 mmol/L NH_4OAc and 2.5 volumes of 100% ethanol, washed in 70% ethanol, and air-dried. The pellet was resuspended in TE (10 mmol/L Tris-HCL, pH 8.0, 1 mmol/L EDTA) and incubated for 1 hour at 55°C. If DNA was not completely dissolved, the TE incubation process was repeated as

necessary. DNA concentration was determined and dilutions were made with TE to a final concentration of 0.1 $\mu\text{g}/\mu\text{l}$. 500 μl (50 μg DNA), or 100 μl (10 μg DNA) were aliquoted for distribution to the participating laboratories.

Samples from 13 formalin-fixed paraffin-embedded lymphoma/leukemia specimens were also distributed. These included 11 paraffin samples that corresponded to frozen cell specimens (5 B-lineage: 4 follicular lymphoma and 1 chronic lymphocytic leukemia/small lymphocytic lymphoma; 6 T-lineage: 4 peripheral T cell lymphoma, 1 lymphoblastic lymphoma and 1 mycosis fungoides, Sézary syndrome), and two additional paraffin-embedded specimens (1 B-lineage: mantle cell lymphoma; 1 T-lineage: peripheral T cell lymphoma). For paraffin-embedded tissues, four or five 10- μm sections of each block were distributed in tubes to each participating laboratory and DNA extraction was performed in the individual laboratories. Laboratories were not aware that that some frozen and paraffin samples were from the same tumor.

Results were compared between laboratories with the exception of Southern blot analysis for rearrangements of the T cell receptor γ chain gene (*TCR γ*), which was performed in one laboratory for comparison to the polymerase chain reaction testing for rearrangements of this locus. For *TCR γ* Southern blot, 16 μg of extracted DNA was digested with *HindIII* and *EcoRI* for 3 hours. The digested DNA was loaded on a 0.7% agarose gel and run at 34 V for 16 hours at room temperature. After electrophoresis, the DNA was transferred overnight with 0.4 N NaOH onto a nylon membrane. The blots were hybridized with H60 probe (ATCC 59585, Rockville, MD).⁷ H60 probe was labeled with P-32 nucleotides by use of a random primer labeling kit (Amersham Pharmacia, Arlington Heights, IL). The blots were washed with 0.1X SSC and 0.1% SDS, and exposed to X-ray films for 5 days. The presence of additional bands or loss of bands when compared to a germline control was considered evidence of a *TCR γ* gene rearrangement.

The submitting laboratory, using their own established guidelines, interpreted the test results. Specific guidelines for interpretation were not proposed by the exchange.

Samples were accompanied by a technical questionnaire related to the individual test performed and laboratories were requested to return the completed questionnaire, a summary of results and copies of diagnostic radiographs and/or gels used for interpreting results.

χ^2 analysis of probability was performed using GB-STAT version 7.0 (Dynamic Microsystems, Inc., Silver Spring, MD). Probability (*P*) values of 0.05 or less were considered statistically significant.

Results

Results were obtained from 24 laboratories, including one laboratory that performed *TCR γ* PCR by two different methods and was counted twice to give a total of 25 laboratory results. Thirteen laboratories performed Southern blot analysis for TCR gene rearrangements on some

or all of the extracted DNA specimens, two performed *TCR β* PCR, and 21 performed *TCR γ* PCR.

Technical Questionnaire

The technical questionnaire for TCR Southern blot analysis indicated that laboratories routinely used two to five different restriction enzymes with the majority of laboratories (9 of 13) using *Bam*HI, *Eco*RI, and *Hind*III. Of the 11 laboratories responding, three required only one rearranged band for a positive result and the remaining eight required two rearranged bands before interpreting a case as rearranged. Seven laboratories used isotopic probes labeled in their own laboratory and seven used non-isotopic methods. Six laboratories used *J β_{1-11}* probes only, four used *CT β* probes only and three used both.

The technical questionnaire on *TCR γ* PCR indicated that paraffin section extraction methods varied from organic in 7 (33%), inorganic in 7 (33%), and crude lysate in 5 (24%) laboratories. No information was received from two (10%) of the participating laboratories. Twelve centers (57%) used a "hot start" PCR approach and 8 (38%) used standard PCR. One center gave no information regarding the PCR approach. All 20 responding laboratories used a non-nested PCR method, but the *TCR γ* variable regions covered by the primers varied. Nine laboratories (43%) used a combination of primers directed against all 11 *V γ* regions (*V γ* 1–8, 9, 10, 11) as well as a multiplex of *J γ* primers (Group 1);^{8–11} 4 centers (19%) used a *J γ* multiplex with primers directed against *V γ* 1–8, 10 and 11 (Group 2);^{12–15} 1 laboratory (5%) used primers directed against *V γ* 1–8, and 9 with a multiplex of *J γ* primers (Group 3);⁸ and, 5 (25%) used a single set of primers directed against *V γ* 1–8 and *J γ* (Group 4).^{16–19} The *V γ* regions covered by the PCR primers were not available for 2 laboratories. The *J γ* primers used were variable within the groups. In Group 1, two laboratories used a multiplex of *J1*, *JP*, *JP1* and *JP2*; 1 laboratory each used the following combinations: *J2*, *JP*, *JP1* and *JP2*; *J1* and *J2*; *J2* and *JP2*; *J1/2* and *JP*; *J1/2*, *JP*, *JP1* and *JP2*; and *J1/2* and *JP1*. Two laboratories in Group 2 used multiplex of *J2* and *JP2*, while the other 2 laboratories in this group used the combinations of *J2*, *JP* and *JP2*, and *J2*, *J1/2* and *JP2*. The single laboratory in Group 3 reported use of a multiplex of *J2*, *JP*, and *JP1*. Three of the laboratories in Group 4 reported use of a single set of *J2* primers, one used a single set of *J1/2* primers and specifics of the single primer set used were not provided by one laboratory.

PCR products were analyzed on polyacrylamide gel in 14 laboratories (66%), by capillary electrophoresis in 3 (14%) and by agarose, MetaPhor (Cambrex, East Rutherford, NJ), and denaturing gradient gel electrophoresis (DGGE) in 1 laboratory each. The method of analysis was not reported for one laboratory. Denaturing conditions (heteroduplex) were used in 6 of the 20 laboratories with data. Ethidium bromide staining was used by 12 of 19 reporting labs, by silver staining in 2 labs, by SYBR green staining in 2 laboratories, and by Gelstar, incorporated radionucleotide and chemiluminescence in 1 laboratory each.

Types of controls included patient samples (9 laboratories; 43%) and cell lines (10 laboratories; 47%). Types of controls were not reported for 2 laboratories (10%). Fifteen laboratories gave information on the type of sensitivity controls that they used, with diluted cells used by 3 and diluted DNA by 12. The range of sensitivities predicted by the laboratories was 0.001 to 10% for frozen samples and 0.01 to 10% for paraffin samples. Laboratories predicted that their *TCR γ* methodology could detect 75 to 95% of clonal T cell rearrangements with a mean of 86%.

TCR γ Southern Blot

Southern blot analysis showed rearrangement of the *TCR γ* gene in all cases of T cell lymphoma and none of the B cell neoplasms.

Exchange Results

The Southern blot results are summarized in Table 1. Although all laboratories that performed Southern blot analysis for TCR gene rearrangements did not test every specimen, presumably due to a lack of sufficient DNA for all tests covered by the sample exchange, there was concordance among all testing laboratories on 10 of 14 samples. The complete concordance was on four B cell neoplasms (all *TCR β* germline) and six T cell neoplasms (all *TCR β* rearranged). Differences in results in four cases included one follicular lymphoma (eight laboratories *TCR β* rearranged and two germline), one mycosis fungoides/Sézary syndrome (11 laboratories *TCR β* rearranged, one germline), and two peripheral T cell lymphomas (one case with 4 laboratories *TCR β* rearranged and three germline; one case with 12 laboratories *TCR β* rearranged and one germline). For the 5 laboratories with a discordant result, no trend related to probe type was identified when compared to the overall results. Two used *CT β* probes and three used *J β_{1-11}* . Two of the laboratories with discordant results used isotope-labeled probes and 3 used non-isotopic probes. No discordant results were reported from the 3 laboratories that used both *CT β* and *J β_{1-11}* probes, although 1 of those laboratories only tested 3 of the 14 available samples. Based on the consensus of the participating laboratories of no *TCR β* rearrangements detected 6 of 7 of the B cell neoplasms, and apparent dual T and B cell rearrangements in one B cell lymphoma, the results of the survey were interpreted as representing no false positive results for *TCR β* Southern blot analysis.

Polymerase chain reaction testing for *TCR β* was only performed in two laboratories, limiting the evaluation of this assay as a diagnostic test. Table 2 summarizes the results from these laboratories, compared to the consensus Southern blot results for *TCR β* (including one *TCR β* -positive B cell neoplasm). Only 47.1% of expected *TCR β* rearrangements were detected by this method with a 15% false positive rate.

Polymerase chain reaction testing for *TCR γ* was performed in 21 laboratories, and the results are summa-

Table 1. Summary of Southern Blot Results

Sample no.	Lineage and diagnosis	J _H	TCR γ	TCR β
1	B-chronic lymphocytic leukemia	R	G	10/10 (100%)G
2	B-chronic lymphocytic leukemia	R	G	10/10 (100%)G
3	T-lymphoblastic lymphoma	G	R	12/12 (100%)R
4	B-follicular lymphoma, grade I	R	G	8/10 (80%)R 2/10 (20%)G
5	T-lymphoblastic lymphoma	G	R	11/11 (100%)R
6	T-mycosis fungoides/Sézary syndrome	G	R	11/12 (92%)R 1/12 (8%)G
7	T-peripheral T cell lymphoma	G	R	4/7 (57%)R 3/7 (43%)G
8	B-follicular lymphoma, grade III	R	G	10/10 (100%)G
9	T-peripheral T cell lymphoma	G	R	12/13 (92%)R 1/13 (8%)G
10	B-follicular lymphoma, grade I	R	G	10/10 (100%)G
11	T-peripheral T cell lymphoma	G	R	7/7 (100%)R
12	B-follicular lymphoma, grade I	R	G	9/9 (100%)G
13	B-follicular lymphoma, grade I	R	G	10/10 (100%)G
14	T-peripheral T cell lymphoma	G	R	12/12 (100%)R

R, rearranged; G, germline.

ized in Tables 2 and 3. Overall, these tests detected 77.9% of expected T cell gene rearrangements. There was a significant decrease in the ability to detect the gene rearrangement in paraffin-embedded tissue, ranging from 85.4% of extracted DNA samples and 65.9% of paraffin samples ($P = 0.0005$). A 4.1% false positive rate was detected, overall, and no difference was seen between paraffin and extracted DNA samples. Almost 22% of samples, however, were not tested, presumably due to

the lack of amplification of an internal control gene. A lack of testing was significantly higher for the paraffin embedded samples (7.1% of extracted DNA samples *versus* 39.6% for paraffin samples; $P < 0.0001$).

The TCR γ results were further analyzed by paraffin extraction methods, PCR method (hot start *versus* standard), DNA analysis method (capillary electrophoresis *versus* polyacrylamide gel and denaturing *versus* non-denaturing gel analysis) and TCR γ variable and joining

Table 2. TCR PCR Results

Sample no.	Lineage and diagnosis	Specimen type	TCR β SB	TCR β PCR	TCR γ SB	TCR γ PCR
1	B-chronic lymphocytic leukemia	Frozen	G	1/2	G	6/18 (33%)
2a	B-chronic lymphocytic leukemia	Frozen	G	0/2	G	0/19 (0%)
2b	B-chronic lymphocytic leukemia	Paraffin	G	ND	G	1/15 (7%)
3	T-lymphoblastic lymphoma	Frozen	R	2/2	R	21/21 (100%)
4	B-follicular lymphoma, grade I	Frozen	R	0/2	G	0/19 (0%)
5a	T-lymphoblastic lymphoma	Frozen	R	1/2	R	21/21 (100%)
5b	T-lymphoblastic lymphoma	Paraffin	R	ND	R	5/9 (56%)
6a	T-mycosis fungoides/Sézary syndrome	Frozen	R	1/2	R	9/20 (45%)
6b	T-mycosis fungoides/Sézary syndrome	Paraffin	R	ND	R	1/8 (13%)
7a	T-peripheral T cell lymphoma	Frozen	R	2/2	R	20/20 (100%)
7b	T-peripheral T cell lymphoma	Paraffin	R	0/1	R	14/17 (82%)
8a	B-follicular lymphoma, grade III	Frozen	G	0/2	G	0/19 (0%)
8b	B-follicular lymphoma, grade III	Paraffin	G	ND	G	0/6 (0%)
9a	T-peripheral T cell lymphoma	Frozen	R	0/2	R	14/21 (67%)
9b	T-peripheral T cell lymphoma	Paraffin	R	ND	R	2/8 (25%)
10a	B-follicular lymphoma, grade I	Frozen	G	0/2	G	0/21 (0%)
10b	B-follicular lymphoma, grade I	Paraffin	G	ND	G	0/10 (0%)
11a	T-peripheral T cell lymphoma	Frozen	R	1/2	R	18/20 (90%)
11b	T-peripheral T cell lymphoma	Paraffin	R	0/1	R	12/16 (75%)
12a	B-follicular lymphoma, grade I	Frozen	G	0/1	G	0/19 (0%)
12b	B-follicular lymphoma, grade I	Paraffin	G	0/1	G	1/15 (7%)
13a	B-follicular lymphoma, grade I	Frozen	G	0/2	G	0/20 (0%)
13b	B-follicular lymphoma, grade I	Paraffin	G	ND	G	0/13 (0%)
14a	T-peripheral T cell lymphoma	Frozen	R	0/2	R	20/21 (95%)
14b	T-peripheral T cell lymphoma	Paraffin	R	ND	R	12/17 (71%)
15	T-peripheral T cell lymphoma	Paraffin	NA	1/1	NA	2/16 (13%)
16	B-mantle cell lymphoma	Paraffin	NA	1/2	NA	2/15 (13%)
17	B-follicular lymphoma, grade I	Frozen	NA	1/2	NA	0/17 (0%)
18	B-follicular lymphoma, grade III	Frozen	NA	0/2	NA	0/16 (0%)

R, rearranged; G, germline; Frozen, extracted DNA from previously frozen samples; ND, not tested; NA, material not submitted for Southern blot testing.

Table 3. Combined Results of All Laboratories for TCR PCR

	Frozen TP	Frozen FP	Frozen not tested	Paraffin TP	Paraffin FP	Paraffin not tested	Total TP	Total FP	Total not tested
<i>TCRβ</i>	7/14 50.0%	2/17 11.8%	1/32 3.1%	1/3 33.3%	1/3 33.3%	20/26 76.9%	8/17 47.1%	3/20 15.0%	21/58 36.2%
<i>TCRγ</i>	123/144 85.4%	6/168 3.6%	24/336 7.1%	60/91 65.9%	4/74 5.4%	108/273 39.6%	183/235 77.9%	10/242 4.1%	132/609 21.7%

A comparison of extracted DNA samples (frozen) to paraffin samples showed a significant increase in true positive results for frozen testing ($P = 0.0005$), and a significant increase in cases not tested for paraffin samples ($P < 0.0001$). No difference in false positive results was observed. TP, true positive; FP, false positive.

regions covered by the PCR primers. These results are summarized in Tables 4-8. The use of inorganic extraction methods of paraffin tissue resulted in a slight, but significant ($P = 0.0059$) increase in false positive results, when compared to organic and crude lysate methods (Table 4). Although a significant difference in results was not observed when hot start PCR was compared to standard PCR, more cases could not be analyzed due to lack of amplifiable DNA when standard PCR methods were used ($P = 0.0053$) (Table 5). When capillary electrophoresis was compared to polyacrylamide gel analysis of DNA products, a slight increase in paraffin false positive results was observed with capillary electrophoresis (2/12 or 16.7% versus 1/53 or 1.9%; $P = 0.0276$) (Table 6). However, significantly fewer cases could be analyzed using polyacrylamide gels (20.2% polyacrylamide gel paraffin cases not tested versus 10.3% for capillary electrophoresis; $P = 0.0316$). Although there was a slight increase in extracted frozen samples not tested when non-denaturing conditions were compared to denaturing gels ($P = 0.0257$), no other significant differences between these methods were identified (Table 7).

Analysis of the different primer sets used for *TCRγ* PCR (Table 8), failed to identify a significant difference in false positive results with any primer group. However, primers strategies devised to amplify *Vγ* 1-8, and 9 (with or

without *Vγ* 10 or 11) with a multiplex of *Jγ* primers (Groups 1-3), had significantly higher true positive rate for extracted frozen cell DNA ($P = 0.0082$), paraffin samples ($P = 0.0027$) and all samples ($P < 0.0001$) when compared to Group 4, which used a single a single set of primers to detect *Vγ* 1-8 and *Jγ*.

Discussion

The results of this sample exchange provide useful information concerning the methodology used and the reproducibility of T cell receptor testing in diagnostic laboratories. When sufficient tissue is available, Southern blot analysis remains a fairly reproducible test. Although some laboratories reported apparent false negative results for this methodology, no methodology or probe-specific trend for false negative results could be identified. The false negative results, however, are disturbing, as this method is generally considered the gold standard for T cell clonality testing. False positive results for T-cell receptor β chain rearrangements are apparently rare with no such examples identified in the exchange. One B cell neoplasm (Case 4) demonstrated rearrangement of both the immunoglobulin heavy chain gene and the T cell receptor β chain gene. Such rearrangements in B cell

Table 4. *TCRγ* Paraffin Extraction Methods

Extraction method	Lab <i>n</i>	TP	FP	Not tested
Organic	7	21/34 (61.8%)	0/30 (0%)	27/91 (29.7%)
Inorganic	7	26/34 (76.5%)	4/23 (17.4%)	34/91 (37.4%)
Crude lysate	6	11/26 (42.3%)	0/26 (0%)	26/78 (33.3%)
No data	2	3/4 (75.0%)	0/1 (0%)	21/26 (80.8%)

Contingency table analysis revealed a significant difference ($P = 0.0059$) when the three methods were compared for false positive results. TP, true positive; FP, false positive.

Table 5. *TCRγ* Hot Start versus Standard PCR

	Lab <i>n</i>	Frozen TP	Frozen FP	Frozen not tested	Paraffin TP	Paraffin FP	Paraffin not tested	Total TP	Total FP	Total not tested
Standard	8	47/56 83.9%	4/58 6.9%	14/128 10.9%	20/31 64.5%	1/27 3.7%	46/104 44.2%	67/87 77.0%	6/85 7.1%	60/232 25.9%
Hot start	12	72/83 86.7%	2/106 1.9%	3/192 1.6%	37/56 66.1%	3/46 6.5%	54/156 34.6%	109/139 78.4%	5/152 3.3%	57/348 16.4%
No data	1	4/5 80.0%	0/4 0%	7/16 43.8%	3/4 75.0%	0/1 0%	8/13 61.5%	7/9 77.8%	0/5 0%	15/29 51.7%

The difference between frozen samples not tested and total samples not tested using standard and hot start PCR methods was statistically significant ($P = 0.0002$ and $P = 0.0053$, respectively). TP, true positive; FP, false positive.

Table 6. TCR γ Gel Analysis

Gel	Lab <i>n</i>	Frozen TP	Frozen FP	Frozen not tested	Paraffin TP	Paraffin FP	Paraffin not tested	Total TP	Total FP	Total not tested
Agarose	1	6/7 85.7%	0/8 0%	1/16 6.3%	0/1 0%	0/2 0%	10/13 76.9%	6/8 75.0%	0/10 0%	11/29 37.9%
Capillary electrophoresis	3	20/21 95.2%	2/26 7.7%	1/48 2.1%	12/19 63.2%	2/12 16.7%	8/39 20.5%	32/40 80.0%	4/38 10.5%	9/87 10.3%
DGGE	1	7/7 100%	0/9 0%	0/16 0%	2/2 100%	1/1 100%	10/13 76.9%	9/9 100%	1/10 10.0%	10/29 34.5%
MetaPhor	1	5/6 83.3%	0/9 0%	1/16 6.3%	3/4 75.0%	0/5 0%	4/13 30.8%	8/10 80.0%	0/14 0%	5/29 17.2%
Polyacrylamide	14	81/98 82.7%	4/112 3.6%	14/224 6.3%	40/61 65.6%	1/53 1.9%	68/182 37.4%	121/159 76.1%	5/165 3.0%	82/406 20.2%
No data	1	4/5 80.0%	0/4 0%	7/16 43.8%	3/4 75.0%	0/1 0%	8/13 61.5%	7/9 77.8%	0/5 0%	15/29 51.7%

Comparison of capillary electrophoresis vs. polyacrylamide gel electrophoresis revealed an increase in paraffin ($P = 0.0276$) and total ($P = 0.043$) false positive results for capillary electrophoresis, as well as an increase in the number of paraffin ($P = 0.0444$) and total ($P = 0.0316$) cases not tested in the polyacrylamide electrophoresis group. TP, true positive; FP, false positive; DGGE, denaturing gradient gel electrophoresis.

malignancies are not uncommon, reportedly occurring in 15% of B cell tumors.²⁰

Few conclusions can be drawn from the results of the TCR β polymerase chain reaction sample exchange due to the small number of laboratories performing this test. The results suggest, however, that the methods used by participants in this exchange for detecting TCR β by PCR fail to detect a significant number of cases, with just over half of the T cell neoplasms detected in this study.

More information can be drawn from the TCR γ PCR sample exchange. Testing for rearrangement of the TCR γ gene by PCR analysis has been increasingly popular in diagnostic laboratories. This gene is rearranged earlier in

T cell development than the TCR α and β genes, and therefore TCR γ gene rearrangements are detectable in virtually all T cell neoplasms.^{4,10,11} In addition, the number of variable and joining regions of TCR γ is less than for TCR β , with only four variable region families made up of a total of 11 variable loci and 5 joining region loci. PCR testing directed at the four variable region families with a multiplex of joining region primers can theoretically detect all TCR γ rearrangements.² The results of the exchange highlight the extreme variability in the methods used for this test. This test is performed well in many laboratories, but several laboratories had suboptimal results when testing paraffin embedded tissues. All labora-

Table 7. TCR γ Denaturing versus Non-Denaturing Gel Conditions

	Lab <i>n</i>	Frozen TP	Frozen FP	Frozen not tested	Paraffin TP	Paraffin FP	Paraffin not tested	Total TP	Total FP	Total not tested
Denature	6	37/42 88.1%	3/53 5.7%	1/96 1.0%	16/24 66.7%	2/17 11.8%	37/78 47.4%	53/66 80.3%	5/70 7.1%	38/174 21.8%
Non-denature	14	82/97 84.5%	3/111 2.7%	16/224 7.1%	41/63 65.1%	2/56 3.6%	63/182 34.6%	123/160 76.9%	5/167 3.0%	79/406 19.5%
No data	1	4/5 80.0%	0/4 0%	7/16 43.8%	3/4 75.0%	0/1 0%	8/13 61.5%	7/9 77.8%	0/5 0%	15/29 51.7%

A significant increase in frozen samples not tested was identified when non-denaturing conditions were compared to denaturing conditions ($P = 0.0257$). TP, true positive; FP, false positive.

Table 8. TCR γ Primer Groups

Group	Lab <i>n</i>	Frozen TP	Frozen FP	Frozen not tested	Paraffin TP	Paraffin FP	Paraffin not tested	Total TP	Total FP	Total not tested
1	9	57/62 91.9%	2/77 3.7%	5/144 3.5%	27/38 71.1%	3/32 9.4%	47/117 40.2%	84/100 84.0%	5/109 4.6%	52/261 19.9%
2	4	26/28 92.9%	1/27 3.7%	9/64 14.1%	14/17 82.4%	1/14 7.1%	21/52 40.4%	40/45 88.9%	2/41 4.9%	30/116 25.9%
3	1	6/7 85.7%	0/8 0%	1/16 6.3%	4/4 100%	0/2 0%	7/13 53.8%	10/11 90.9%	0/10 0%	8/29 27.6%
1+2+3	14	89/97 91.8%	3/112 2.7%	15/224 6.7%	45/59 76.3%	4/48 8.3%	75/182 41.2%	134/156 85.9%	7/160 4.4%	90/406 22.2%
4	5	26/35 74.3%	3/43 7.0%	2/80 2.5%	9/22 40.9%	0/20 0%	23/65 35.4%	35/57 61.4%	3/63 4.8%	25/145 17.2%
No Data	2	8/12 66.7%	0/13 0%	7/32 21.9%	6/10 60.0%	0/6 0%	10/26 38.5%	14/22 63.6%	0/19 0%	17/58 29.3%

Contingency table analysis of Groups 1–3 versus Group 4 revealed significant difference for Frozen TP ($P = 0.0082$), Paraffin TP ($P = 0.0027$) and Total TP ($P < 0.0001$). TP, true positive; FP, false positive.

tories estimated in the technical survey that they could detect at least 75% of T cell neoplasms, with many reporting a 95% expected detection. Despite this, the overall detection rate in paraffin tissues was 65.9% in this study. This suggests that a significant number of laboratories have not actually compared their results for paraffin and fresh/frozen samples and may not realize the potential risk of false negative results on paraffin tissues. Difficulty in extracting sufficient DNA for analysis from the paraffin tissue is the most likely cause for this drop in detection. This difficulty is also suggested by the significantly higher number of paraffin samples that were not tested in the exchange, presumably due the inability to extract sufficient DNA for analysis and the resulting failure to amplify an internal control gene. An increase in the inability to test samples was also related to use of standard PCR (in comparison to hot start PCR), use of non-denaturing gel conditions, and use of polyacrylamide gels (compared to capillary electrophoresis). These results suggest that improved extraction methods, hot start PCR methods and more sensitive detection methods may reduce the number of false negative results for this test.

False positive results for *TCR γ* PCR were not common in the sample exchange, occurring at a rate of 3.6% for extracted frozen tissue DNA, 5.4% for paraffin samples and 4.1% overall. One parameter identified to be related to an increase in false positives was use of inorganic extraction methods on paraffin embedded tissues. Although these methods yielded a slight increase in true positive results (76.5%) when compared to organic (61.8%) and crude lysate (42.3%) methods, they also resulted in a slight, but statistically significant increase in false positive results. The use of capillary electrophoresis was also associated with an increase in false positive results when compared to the use of polyacrylamide gel electrophoresis. Therefore, although the use of this newer methodology appears to increase the detection of rearrangements, it may also introduce an increase risk for false positive results. The increase in false positive results with capillary electrophoresis could be related to over-interpretation of results with this very sensitive methodology. As more laboratories begin using this method of detecting *TCR γ* rearrangements, the potential for false positive results should be recognized and stringent interpretative criteria should be developed to reduce this possibility.

An increase in false positive *TCR γ* PCR has been previously reported with the use of primers directed against *V γ 9*. Some circulating γ/δ T cells reportedly undergo normal polyclonal gene rearrangements with little junctional diversity. These so-called canonical *TCR γ* rearrangements do not demonstrate nucleotide additions and therefore may produce polyclonal rearrangements that are very similar or identical in size. This phenomenon is reported to occur more frequently with rearrangements involving *V γ 9* and *J γ P*.²¹ No such increase in false positive results was identified in this study in Groups 1–3, which included primers directed against the *V γ 9* region and all of the *J γ* regions of the *TCR γ* gene.

The primer strategy used to detect *TCR γ* rearrangements by PCR analysis was a highly important factor in

the ability of laboratories to detect rearrangements on a consistent basis. Laboratories that used a single set of primers directed against consensus regions of *J γ* and *V γ 1–8* had the lowest rate of detection. The addition of more primers sets, including primers to cover all *J γ* regions and some or all of the other *V γ* regions (*V γ 9*, 10, 11), significantly increased the ability to detect *TCR γ* gene rearrangements without increasing the rate of false positive results. The cases tested in this exchange were randomly chosen without consideration of *V γ* region rearrangements.

Recently, a multicenter trial of German, Austrian, and Swiss investigators reported their results for *TCR γ* PCR testing.²² In that study, 26 laboratories studied six paraffin samples, including four T cell neoplasms and two reactive hyperplasias. Overall, an average of 90% of *TCR γ* results was considered “correct” in that study, with correct results ranging from 69% to 100%. Further details of the results were limited in that report. Although the German-Austrian-Swiss study results suggest a higher detection rate than the results of the current study, the small number samples and the lack of detailed results for the former study limits the comparison of the two multicenter studies.

Recent attempts to standardize PCR testing have also been published. European laboratories have developed standardized primers and protocols for the detection of minimal residual disease in acute lymphoblastic leukemia, which include testing for *TCR γ* .²³ The reproducibility of this approach, however, has not yet been reported.

In summary, this sample exchange for T cell receptor testing provides a large amount of data, but several key points can be made: 1) Southern blot analysis for *TCR β* chain gene rearrangements remains a reliable and reproducible test with minimal false positive results. 2) *TCR γ* PCR testing is also a reliable and reproducible test that is performed well in many laboratories, especially on fresh or frozen tissues. 3) Paraffin sample *TCR γ* PCR testing is less reliable than fresh or frozen tissue testing in most laboratories, and the risk for false negative results for paraffin tissues should be recognized. 4) A high number of samples, particularly paraffin tissues, could not be reliably tested due to a lack of amplifiable DNA. This appears to be related to a variety of technical factors. 5) Single primer set strategies that are only directed against a consensus of the *V γ 1–8* and *J γ* regions of *TCR γ* are suboptimal for the detection of many clonal T cell neoplasm samples.

Based on the findings of this exchange, three recommendations can be made. First, the authors continue to support previously published criteria for the interpretation of Southern blot studies that require the identification of two rearrangements (either two rearrangements in one digest or one rearrangement in two different digests).²⁴ Second, PCR testing for *TCR γ* gene rearrangements should employ primers directed against all areas of the *TCR γ* V and J regions to minimize false negative results. Finally, laboratories that wish to perform testing in paraffin-embedded tissues should separately verify their methodology on that tissue type.

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