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ORIGINAL ARTICLE

Translocation detection in lymphoma diagnosis by split-signal FISH: a standardised approach

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Abstract Lymphomas originating from the lymphatic system comprise about 30 entities classified according to the World Health Organization (WHO). The histopathological diagnosis is generally considered difficult and prone to mistakes. Since non-random chromosomal translocations are specifically involved in different lymphoma entities, their detection will be increasingly important. Hence, a split-signal fluorescence in situ hybridisation (FISH) procedure

In memoriam: Prof. David Mason died in February 2008.

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would be helpful in discriminating the most difficult classifications. The Euro-FISH programme, a concerted action of nine European laboratories, has validated a robust, standardised protocol to improve the diagnostic approach on lymphoma entities. Therefore, 16 fluorescent probes and 10 WHO entities, supplemented with reactive cases, were selected. The results of the Euro-FISH programme show that all probes were correctly cytogenetically located, that the standardised protocol is robust, resulting in reliable results in approximately 90% of cases, and that the procedure could be implemented in every laboratory, bringing the relatively easy interpretation of split-signal probes within the reach of many pathology laboratories.

Keywords Split-signal FISH · Lymphoma · Validation · Classification · Chromosomal aberration

Introduction

The Euro-FISH project represents a concerted multi-centre initiative in the field of lymphoma diagnosis. These cancers, which originate from the immune system, differ widely in their clinical behaviour and in terms of the therapy needed.

The diagnosis of lymphomas can be a complex process, which needs to take into account clinical, morphological, immunophenotypic and genetic features. Different lymphoma types are associated with non-random chromosomal translocations (Table 1) and the detection of these aberrations is a fundamental step in the identification of the different entities.

For example, Burkitt lymphoma (BL), a highly aggressive lymphoma, is associated with a translocation involving c-*myc* gene in more than 90% of cases [1]. In over 95% of mantle cell lymphomas (MCL), a t(11;14)(q13;q32) is found involving the *cyclin D1* and *IGH* genes [2]. In addition, a translocation of the *BCL2* gene to the *IgH* gene locus resulting in a t(14;18) is a hallmark of follicular lymphoma [3] and is only seen in 20–30% of the diffuse large B cell lymphoma (DLBCL) IGH translocations. Furthermore, in DLBCL, 14% show 3q27 aberrations and 14% display MYC rearrangement (Mitelman Database of Chromosome Aberrations in Cancer 2006; http://cgap.nci.nih.gov/Chromosomes/Mitelman).

Moreover, some cytogenetic alterations define clinically relevant subgroups and are, therefore, crucial for therapy decisions. For instance, gastric marginal zone lymphomas (gastric MALT lymphomas) lacking the t(11;18) involving the *MALT1* gene respond to *Helicobacter pylori* eradication therapy in contrast to the t(11;18)-positive cases that do not respond [4].

Based on these and other available data, it is of increasing importance to know the underlying recurring chromosomal aberrations. In this way, initial correct patient-tailored therapy can be given, preventing over- or under-treatment.

At present, these cytogenetic abnormalities are not easily detected in the routine laboratory. Cytogenetic analysis, based on banding techniques, will present an overview of all cytogenetic aberrations. However, lack of success in culturing tumour cells, low mitotic indices and the lack of fresh material often complicates the use of this technology for routine diagnosis. In a recent review [5], it was nicely outlined that fluorescence in situ hybridisation (FISH) has, over the last decade, become a firmly established technique.

To detect a translocation in a tumour cell, one can use probes with different colours on different chromosomes (usually two) in such a way that, in the case of a translocation, a fusion signal occurs (Fig. 1a). This procedure is feasible in cytospins or preparations of isolated nuclei, but more difficult in tissue sections where many nuclei are cut and thus a complete signal is present in a minority of cells,

Table 1 Selection of lymphoma entities and frequently found translocations (as taken from the World Health Organization Classification of Tumours, Pathology and Genetics, Tumours of Haematopoietic and Lymphoid Tissues by Jaffe [13])

Selected entity	Associated translocation	Percentage
Diffuse large B cell lymphoma (DLBCL)	t(14;18) (IGH; 14q32) (BCL2;18q21) and 3q27 abnormalities (BCL6)	20–30 30
Mantle cell lymphoma (MCL)	t(11;14)(q13;q32) (cyclin D1; 11q13) (IGH; 14q32)	70–75
Lymphocytic leukaemia (B-CLL/SLL)	t(14;18) (q32;q21) (IGH; 14q32) (BCL2;18q21)	5–10
Follicular lymphoma (FCL)	t(14;18) (q32;q21) (IGH; 14q32) (BCL2;18q21)	70–95
Gastric MALT	t(11;18)(q21;q22) (MLT; 18q22)	30
Splenic marginal zone lymphoma (MZL)	Allelic loss 7q21–32	40
Burkitt lymphoma (BL)	t(8;14)(q24;q32) (MYC; 8q24)	100
Lymphoplasmacytic lymphoma (LPL)	t(9;14)(p13;q32) (pax5; 9p13)	5
Anaplastic large cell lymphoma (ALCL)	t(2;5)(p23;q35) (ALK; 2p23)	70
T lymphoblastic lymphoma (T-LBL)	T cell receptor alpha and delta (14q11) (TCRAD) beta 7q35 (TCRB), gamma 7p14 (TCRG) (T cell receptor loci, approximately 33%) TCL1	30

making interpretation cumbersome. Split-signal or break-apart probes use differently coloured probes on both sides of a known breakpoint region, resulting in a fused signal in the normal situation, but single colours when a break in the gene occurs (Fig. 1b). This approach is advantageous in tissue sections since each single coloured signal indicates a specific chromosomal break.

The Euro-FISH programme consisted of three stages: probe validation, protocol development and testing, application of the protocol to be used throughout Europe and was started in order to validate a robust, standardised FISH protocol using split-signal probes on formalin-fixed, paraffin-embedded tissue sections. The validity of the diagnostic approach on selected entities of lymphomas was also evaluated. This standardised protocol, when implemented, will be a useful tool in discerning the different types of lymphoma and available treatment options.

Materials and methods

The Euro-FISH project was organised into three stages: Stage 0, probe validation on metaphase slides of B lymphocytes of healthy donors. Stage 1, robustness of the standardised Euro-FISH protocol. In this stage, the Euro-FISH protocol was tested in each laboratory on a laboratory-specific tissue microarray (TMA). Stage 2, the evaluation of the FISH protocol throughout Europe. The Euro-FISH protocol was tested on 144 cases, on four TMAs with all entities and laboratories equally represented.

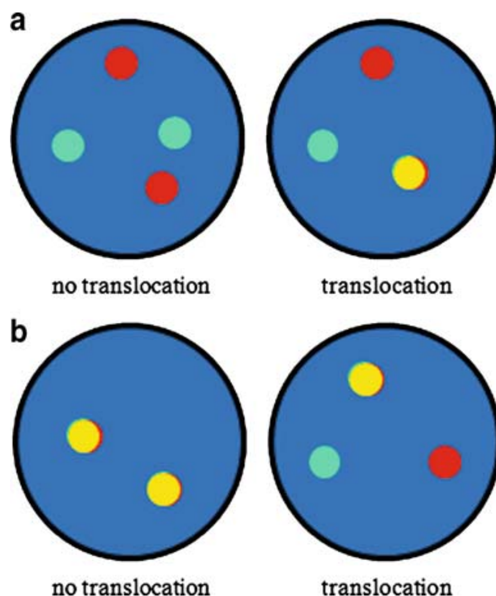

Fig. 1 Schematic representation of nucleus stained with a fusion probe (**a**) and a split-signal probe (**b**) to detect a chromosomal translocation in a tumour cell. **a** *Red* and *green* need to co-localise to detect a known translocation (*right-hand side*) whereas a split-signal probe (**b**) detects a break without the need to know the translocation partner (*right-hand side*)

The evaluation involved testing of 16 FISH probes on 10 different World Health Organization (WHO) lymphoma entities; diffuse large B cell lymphoma (DLBCL), mantle cell lymphoma (MCL), lymphocytic leukaemia (B-CLL/SLL), follicular lymphoma (FCL), gastric marginal zone lymphoma (gastric MALT), splenic marginal zone lymphoma (splenic MZL), African and non-African BL, lymphoplasmacytic lymphoma (LPL), anaplastic large cell lymphoma (ALCL), all ALK+ and T lymphoblastic lymphoma (T-LBL), supplemented with reactive tissues (spleen, tonsil, lymph node and thymus). All samples were neutral-buffered, formalin-fixed.

Probes and FISH procedures

Split-signal FISH probes (Table 2), Histology FISH Accessory Kit (code no. K5599), Cytology FISH Accessory Kit (code no. K5499), Dako Hybridiser system (code no. S2451), Whirlpool JT356 or JT359 microwaves and metaphase slides of B lymphocytes (DR2524 lot:20050627mem) of healthy donors were supplied by Dako (Dako Denmark A/S, Produktionsvej 42, DK-2600 Glostrup, Denmark). All fluorescent microscopes were equipped with microscope-specific double filters (XF53, Omega Optical, Brattleboro, VT, USA) suitable for the fluorescein isothiocyanate- and Texas red-labelled split-signal probes. Paraffin-embedded, neutral-buffered, formalin-fixed biopsies were used in stages 1 and 2. Probes used during the Euro-FISH procedure with corresponding cytogenetic position and code numbers are listed in Table 2. During stages 0, 1 and 2, each laboratory

Table 2 Split-signal probes with corresponding cytogenetic localisation and Dako code number

Probe	Cytogenetic position	Code no.
BCL10	1p22	Y5418
IGK	2p11	Y5416
ALK	2p23	Y5417
BCL6	3q27	Y5408
TCRG	7p14	Y5420
TCRB	7q34	Y5421
MYC	8q24	Y5410
PAX5	9p13	Y5413
CCND1	11q13	Y5414
TCRAD	14q11	Y5419
TCL1	14q32	Y5426
IGH	14q32	Y5406
MALT1	18q21	Y5409
BCL2	18q21	Y5407
BCL3	19q13	Y5411
IGL	22q11	Y5412

tested four probes resulting in laboratory-independent duplicates. Probes were assigned to the laboratories in such a way that duplicates were in general not generated by the same two laboratories (Table 3). Slides were stained according to the manufacturer's manual.

Probe validation

Each probe was validated by analysing at least five metaphases of B lymphocytes of healthy donors per laboratory. Metaphase slides were stained and mounted by using the Cytology FISH Accessory Kit according to the manufacturer's manual. Chromosomes were identified by inverted 4′,6-diamidino-2-phenylindole (DAPI) staining. All probes were validated in duplicate. Therefore, eight laboratories each validated four probes.

Case selection

From the archives of each participating laboratory, cases were selected for the studies. All protocols for obtaining and studying human tissues and cells were approved by each institution's review board for human subject research. The cases had been diagnosed using classic pathological criteria based on morphology and immunophenotype and molecular criteria. No form of translocation detection had been part of the diagnostic process. A central review was not performed. Stages 1 and 2 TMAs were prepared with the 10 above-mentioned WHO lymphoma entities, supplemented with reactive cases.

Table 3 Probes assigned to the nine laboratories in such way that every probe is tested in duplicate

Probe	Country code							
	DE	DK/GR	ES	FR	IT	NL	PT	UK
CCND1			ES	FR				
BCL2			ES		IT			
BCL3		DK/GR					PT	
BCL6					IT			UK
BCL10	DE			FR				
MYC	DE				IT			
PAX5						NL		UK
MALT1	DE					NL		
ALK1		DK/GR					PT	
TCL1	DE					NL		
IGH				FR				UK
IGK		DK/GR	ES					
IGL		DK/GR					PT	
TCRAD				FR	IT			
TCRB			ES				PT	
TCRG						NL		UK

DE Germany, *DK* Denmark (stage 0), *GR* Greece (stages 1 and 2), *ES* Spain, *FR* France, *IT* Italy, *NL* The Netherlands, *PT* Portugal, *UK* United Kingdom

Tissue microarrays

To prepare TMAs, punch needles of 1 mm were used. For stage 1, laboratories prepared TMAs using tissue blocks from their own institutes, resulting in a laboratory-specific TMA. Stage 2 TMAs were made centrally at the Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands, such that every entity and every institute was equally represented. The position of the cores taken from the tissues, used to prepare the TMAs, was based upon hematoxylin and eosin-stained slides.

Stage 1 TMAs were composed of three or four entities, preferably six cases per entity, per laboratory (see also Table 3) supplemented with DLBCL (three cases per TMA) and reactive tissues (three cases per TMA) resulting in 24 to 30 cores per TMA. The four stage 2 TMAs consisted of 36 cores each, in total 144 cases; 12 African BL, 12 non-African BL, 12 ALCL, 12 B-CLL/SLL, 16 DLBCL, 12 FCL, 12 LPL, 12 gastric MALT, 12 MCL, 12 splenic MZL, 12 T-LBL and eight reactive tissues.

Of both stage 1 and 2 TMAs, 3 μm sections were cut, put on glass slides, stained and mounted using the Histology FISH Accessory Kit according to the manufacturer's manual.

During stage 1, laboratories used their own optimal digestion times. Optionally, a 6-min digestion time (if different from the laboratories own optimal digestion time) was used. In stage 2, the digestion time was set at 10 min for all laboratories.

Scoring of the TMAs was performed according to the Euro-FISH guidelines with respect to morphology (good, intermediate, poor/failure), background (absent, acceptable, excessive/failure), signal intensity (strong, moderate, weak, absent/failure) and actual score (normal=YY or abnormal= YYY/YG/YR/GR/YYR/YYG or any other combination except YY or no score).

Results

Stage 0: probe validation

During stage 0, metaphase slides made from B lymphocytes of healthy donors were used to validate all 16 probes in duplicate. Since eight laboratories each tested four probes, duplicates were independently scored. In order to properly validate the probes, five metaphases per probe per laboratory were analysed. All probes localised to the expected position (Table 1) and no irregularities were found. A selection of the probes validated in this stage is shown in Fig. 2.

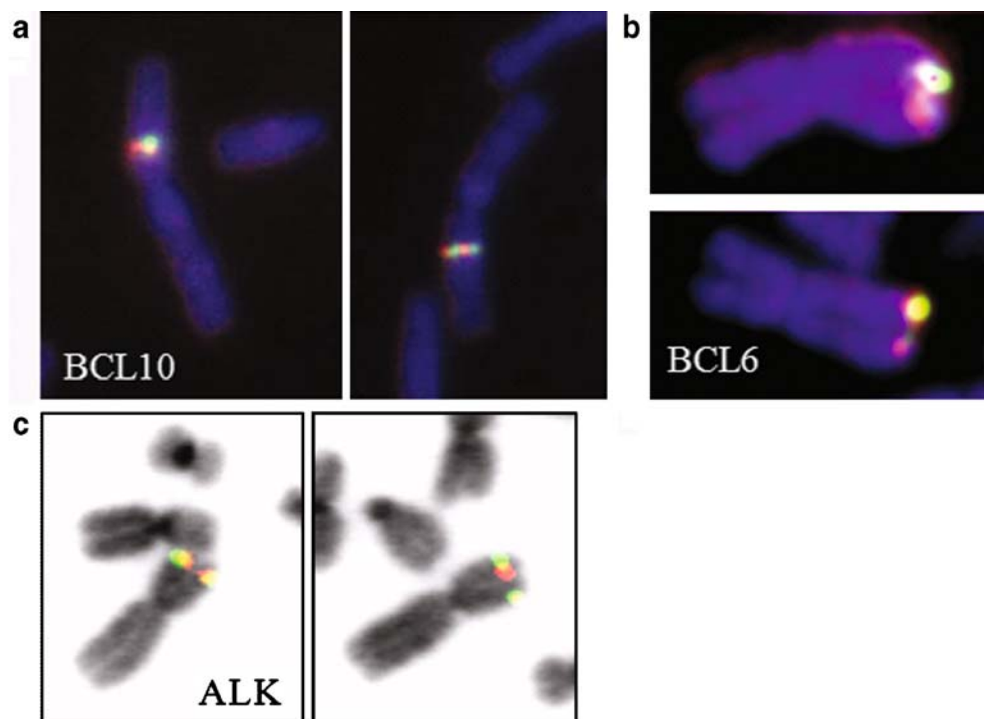

Fig. 2 Selection of probes validated in stage 0. **a** BCL10 localises to chromosomal band 1p22; **b** BCL6 localises to chromosomal band 3q27; **c** ALK localises to chromosomal band 2p23. **a** and **b** A normal DAPI (DNA) fluorescence staining combined with the FISH probe signal. **c** An inverted DAPI staining in combination with the FISH probe signal

Stage 1: robustness of the standardised FISH protocol

To test the robustness of the standardised FISH protocol, FISH testing was optimised in eight laboratories with every laboratory using the same four probes as during stage 0. TMAs used to optimise the protocol were made from laboratories own samples resulting in centre-specific TMAs. Stage 1 TMAs were composed of three or four entities (six cases per entity, if available) per laboratory supplemented with DLBCL (three cases per TMA) and reactive tissues (three cases per TMA) resulting in 24 to 30 cores per TMA. In this stage, laboratories used their own optimal digestion times. Optionally, a 6-min digestion time (if different from the laboratories own optimal digestion time) was performed. In total, 1,096 cores were scored in stage 1. Cores that were lost during the procedure were not taken into account.

From the results of this stage, it is clear that, in different laboratories, slightly different digestion times were needed. In Table 4, results of the laboratory-optimal digestion times are summarised in combination with the percentage of reliable scores, resulting in 91% overall reliable scores (805 cores of which 71 could not be scored). Figure 3 shows the reliability scores of the optimal digestion times per lymphoma entity. African BL cases display a very low overall success percentage of only 60% whereas ALCL, LPL, BL, FCL and B-CLL/SLL all have a score above 90%.

Although digestion times vary from laboratory to laboratory, it became clear that, based on the scoring percentages as summarised in Table 4, a 10-min digestion time was found to be the optimal digestion time and, therefore, used in stage 2.

Considering morphology (data not shown), also scored during stage 1, a higher percentage of nuclei with good morphology was seen using a 10-min digestion time compared with a 6-min digestion time, 86% and 54%, respectively.

Stage 2: the evaluation of the FISH protocol throughout Europe

During stage 2, again eight laboratories each used four probes, providing independent duplicate data per probe. Every laboratory worked with a 10-min digestion time as established in stage 1. Four new TMAs were centrally constructed, such that every laboratory and every entity was equally represented on all four TMAs. Each of the four TMAs included 36 tissue cores. Cores that were lost during the procedure were not taken into account while analysing the data.

Analysing all 144 cores with all 16 probes in duplicate showed that one core was repetitively lost resulting in 143 usable cores. Of these 143 cores, the same 14 cores repeatedly could not be scored reliably in over 50% of the stainings (9.8%), resulting in an overall 90.2% score reliability. Scoring percentages per entity are shown in Table 5.

Results per entity

The results for each entity are given in Table 5. Although, in principle, all samples were fixed and treated in the same way, some lymphoma entities are more easily lost than

Table 4 Stage 1 results

Entity	Country	Digestion time (min)	Cases with score	Percentage score	No.
B-CLL	Spain	6	44	91.67	48
B-CLL	Portugal	10	48	100.00	48
DLBCL	Spain	6	23	95.83	24
DLBCL	Portugal	10	24	100.00	24
DLBCL	Germany	12	11	91.67	12
DLBCL	Netherlands	6	9	75.00	12
DLBCL	France	6	12	100.00	12
DLBCL	Italy	8	12	100.00	12
DLBCL	United Kingdom	5	7	77.78	9
FCL	Spain	6	44	91.67	48
FCL	Italy	8	21	87.50	24
FCL	United Kingdom	5	24	96.00	25
MCL	Spain	6	38	79.17	48
MCL	France	10	5	100.00	5
A-BL	Italy	8	12	60.00	20
BL	Italy	8	7	87.50	8
BL	Germany	12	22	100.00	22
MZL	Italy	8	13	65.00	20
MZL	France	10	5	100.00	5
MZL	United Kingdom	5	28	96.55	29
LPL	United Kingdom	5	20	95.24	21
LPL	Netherlands	6	22	91.67	24
LPL	Germany	12	24	100.00	24
MALT	Netherlands	6	17	85.00	20
MALT	Germany	12	21	87.50	24
MALT	France	10	2	100.00	2
ALCL	Portugal	10	47	97.92	48
T-LBL	Portugal	10	48	100.00	48
T-LBL	Netherlands	6	15	93.75	16
T-LBL	Germany	12	19	79.17	24
Reactive	Spain	6	24	100.00	24
Reactive	Netherlands	6	12	100.00	12
Reactive	Portugal	10	18	75.00	24
Reactive	Germany	12	12	100.00	12
Reactive	Italy	8	9	75.00	12
Reactive	France	10	1	100.00	1
Reactive	United Kingdom	5	14	100.00	14
Total			734	91.18	805

Optimal digestion times per laboratory and per entity. Number and percentages of cores with a score and the total number (No.) of cores scored after optimal digestion per laboratory

others, as shown by comparing gastric MALT lymphoma to non-African Burkitt cases (both types starting with 12 cores×16 probes in duplicate=384 cores) with 89 and 0 lost cores, respectively. Furthermore, the MALT cases appeared to be the most difficult cases to score (82.25% score reliability) whereas B-CLL/SLL resulted in the highest percentage (97.38%) of reliably scored cores. Since the samples, except for the African Burkitt lymphoma cases, were provided by several laboratories (each by three laboratories), laboratory-specific effects can be excluded. African BL, ALCL, LPL, reactive, splenic MZL and T-LBL all resulted in approximately 88% score reliability. B-CLL/SLL, DLBCL, FCL, MCL and non-African BL all show a reliability score of more than 90%.

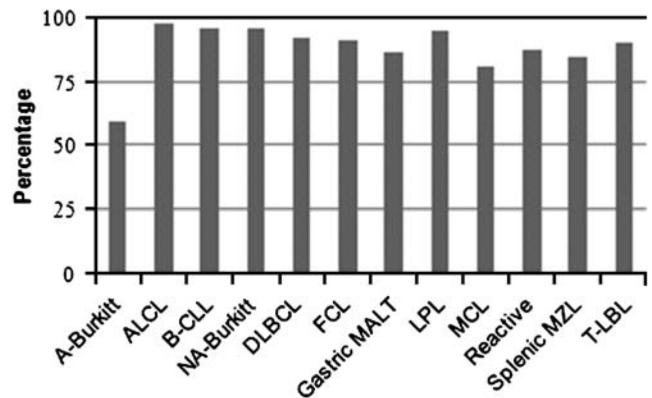

Fig. 3 Reliability scores of the optimal digestion times per lymphoma entity

Table 5 Stage 2, percentage of reliably scored cores per entity

Entity	Digestion time (min)	Total number of cores	Number of cores lost	Number of cores without a score	Percentage of cores without score	Percentage of reliable score	Total percentage
A-Burkitt	10	288	8	34	12.14	87.86	100.00
ALCL	10	320	8	40	12.82	87.18	100.00
B-CLL	10	384	3	10	2.62	97.38	100.00
DLBCL	10	480	13	26	5.57	94.43	100.00
FCL	10	384	3	36	9.45	90.55	100.00
LPL	10	384	7	46	12.20	87.80	100.00
MALT	10	320	89	41	17.75	82.25	100.00
MCL	10	384	6	18	4.76	95.24	100.00
NA-Burkitt	10	384	0	18	4.69	95.31	100.00
Reactive	10	256	32	29	12.95	87.05	100.00
Splenic MZL	10	384	22	41	11.33	88.67	100.00
T-LBL	10	384	39	39	11.30	88.70	100.00
Total		4,352	230	378		90.83	

Lost cores and cores that repeatedly could not be reliably scored in over 50% were not taken into account

Disregarding the African BL cases (due to prolonged fixation), a trend in scorability is noticed with the T cell lymphomas, the indolent B cell lymphomas and reactive cases more difficult to score and the aggressive B cell lymphomas easier to score with FCL taking an intermediate position.

Discussion

Our study shows that FISH analysis for translocations in lymphoma is feasible and reliable on formalin-fixed, paraffin-embedded tissue samples from various laboratories using a variety of probes using a standardised approach. The 16 FISH probes that were tested all localised to the expected position (Table 2). In addition, when every laboratory used their centre-specific TMA and own optimal digestion times in combination with the previously chosen 6-min digestion time, over 90% of tests performed could be scored reliably. This first stage resulted in an overall preferable 6- or 10-min digestion time and the latter was chosen for stage 2. To get a higher success rate, the pepsin digestion time can of course be changed on difficult cases.

As well as during stage 1, during stage 2, when samples obtained from all laboratories and all lymphoma types supplemented with reactive cases were equally distributed, over 90% of all cases could be scored reliably using the Euro-FISH protocol, rendering this technique very useful for routine diagnostics. An exception were cases of African Burkitt lymphoma in which the success rate was lower, probably due to the prolonged fixation of these cases that originated from Africa and had been send to Italy for processing. Of note, the samples were represented on tissue microarrays that contained cores from different laboratories, underscoring the robustness of the protocol.

It is of increasing importance to assess the presence of a chromosomal translocation in the diagnosis and classification of lymphomas, especially, since with ongoing improvement of patient-tailored therapy one needs to prevent over- or under-treatment. As we and others have shown, split-signal FISH is a very fast, reliable and easy-to-use technique to determine whether a break is present in the gene of interest. Subsequently, by using other split probes, fusion probes or split probes for the *V* genes, it is possible to determine the translocation partner. A major advantage of the split-signal probes, as used in our Euro-FISH project, is that translocations can be made visible which cannot be detected by other means. For example, in cases of the *MLL* gene in leukaemia, cytogenetic information cannot be detected by polymerase chain reaction (PCR) due to many (50) possible translocation partners [6]. Furthermore, breakpoints spread over a large genomic region, as is the case for the *CCDN1* gene [7], cannot also be easily detected by PCR analysis. In addition, FISH detection of breakpoints on paraffin-embedded material is very useful if neither frozen material nor fresh material is available for classical cytogenetic analysis or if no usable results can be obtained by classical cytogenetics. To illustrate the power of the Euro-FISH protocol, two case reports are discussed briefly below.

Case 1

A patient, diagnosed with FCL, showed a cytogenetic aberration of chromosome 14 which was documented as add(14)(q32). No classical t(14;18) was found by classical cytogenetics. However, FISH analysis showed that this

patient had a classical t(14;18)(q32;q21) break with only the derivative 14 present. This break was later confirmed by PCR analysis (data not shown).

Case 2

Based on conventional and immunohistochemical stainings, a second patient was diagnosed with a lymphoma of which the classification was not sure. FISH analysis on paraffin-embedded material showed a break for both IgH and BCL2. Based on these results, the routine staining was repeated and confirmed the FCL diagnosis already proven by FISH analysis (data not shown).

In addition to achieving its goal, this study highlighted some unexpected results. Previously, it was thought that 100% of African BL cases carried the t(8;14)(q21;q32) translocation [8, 9]. However, we have found that this is not the case and have identified, in addition to the already known translocation partner, additional partners for chromosome 8 (Leoncini et al., manuscript submitted, J Pathol). Further research will establish whether different translocations in BL will need different treatments.

Besides the above-mentioned importance of knowing the translocation partners, it is also necessary to know whether other chromosomal aberrations are present. Starostik and co-workers [10] suggested that t(11;18)-negative MALT lymphoma, showing numerous allelic imbalances, some of them identical to aberrations seen in DLBCL, would eventually transform into high-grade DLBCL. In addition, deletions of 6q, common in FCL and DLBCL, are associated with adverse clinical behaviour [11, 12].

An analysis of the effect of a lymphoma pathology panel revealed that, when all lymphoma diagnoses are centrally reviewed, 16% of lymphoma patients were incorrectly diagnosed. In these cases, the revised diagnosis influenced the treatment of the patient (van Rijk et al., manuscript in preparation). These incorrect diagnoses could have been prevented in 50% of the cases by the use of FISH analysis (own data). We, therefore, propose that FISH analysis be performed routinely in cases in which the classification is not completely sure. Our data indicate that translocation detection is now a mature method that can be implemented in every department of pathology.

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Conflict of interest Except for Dr. Hauge Matthiesen and Dr. Svenstrup Poulsen who are directly employed at Dako A/S, Denmark, none of the above-mentioned authors have any conflicts of interest.

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