IMMUNOPHENOTYPING USER GUIDE

The Immunophenotyping Laboratory in the Haematology Department at the University Hospital of Wales, Cardiff, provides a flow cytometry and Immunophenotyping service to many hospitals and healthcare organisations in Wales.

The laboratory aims to provide a quality service, and offers a comprehensive portfolio of pathology tests, which use flow cytometry instrumentation to diagnose haematological disease and provide accurate enumeration of specific cell populations in a variety of different sample types and therapeutic products.

There is close collaboration with the following departments:

1. Cytogenetics and Molecular Diagnostics units in Medical Genetics; making a significant contribution towards an integrated diagnostic approach to haematological malignancy testing. In most cases this leads to the production of an integrated report, which incorporates Morphology, Immunophenotyping, Cytogenetics, FISH and PCR (molecular) results.
2. Cellular Pathology, UHW. The laboratory tissue flow cytometry service was suspended during the Covid-19 pandemic, and is currently awaiting review ahead of any renewal.
3. The laboratory also works closely with the Bone Marrow Transplant Unit (BMTU) and Stem Cell Processing Unit (SCPU), providing a timely service for CD34 and/or CD3 enumeration and cell viability assessment, which enables efficient time critical service delivery by the SCPU.

The following user guide has been produced to provide basic information about the Immunophenotyping service, and to aid medical staff in requesting the appropriate tests. Please contact the laboratory for further information.

Contact Details

Contact address:	Immunophenotyping Laboratory, Upper Ground Floor, C Block University Hospital of Wales Heath Park Cardiff, CF14 4XW
Telephone:	029 21842370 (answer machine available)
C&V internal extension	42370
Tie line:	0172 42370

Main Laboratory Contacts

Head of Immunophenotyping Laboratory: Steve Couzens
Tel: 029 21846720, internal ext. 46720
Steve.Couzens@wales.nhs.uk

Deputy: Ian Phillips
Tel: 029 21843458, internal ext. 43458
Ian.Phillips4@wales.nhs.uk

Preferred method of contact: email to one of the above

Main Haematology Department Contacts

Clinical Head of Haematology Laboratory Services: Dr A.P. Goringe
Telephone secretary on 029 20182033, internal ext. 42033

Haematology Services Manager: Alun Roderick
Tel: 029 20184202, internal ext. 44202
Alun.Roderick@wales.nhs.uk

Accreditation and Quality

The Haematology Department is currently UKAS accredited, fully meeting the requirements of ISO15189:2012. The Immunophenotyping laboratory operates internal QC procedures to maintain the quality of its results and participates in the following UKAS accredited NEQAS schemes:

1. Leukaemia Diagnostic and Interpretation (parts 1 and 2)
2. CD34 Stem cell Enumeration
3. Immune Monitoring
4. Paroxysmal Nocturnal Haemoglobinuria
5. Foeto-maternal haemorrhage
6. B-ALL MRD flow cytometry

Details of the laboratory performance are available on request.

Standard Hours of Operation

Monday to Friday	8.45am to 5.15pm
Weekends and Bank Holidays	No routine service

No routine Immunophenotyping service is provided out of hours, at weekends, or on Bank Holidays. Please contact the laboratory in advance for urgent specimens and/or those requiring special attention (such as Hereditary Spherocytosis screening).

Sampling

Flow cytometry requires a single cell suspension, which can be derived from a number of different tissues or serous fluids where involvement with malignant cells is suspected. Blood and bone marrow aspirates (BMA) should be anticoagulated with EDTA. If it is difficult to provide a BMA for analysis (e.g. packed marrow, or BM fibrosis), an **unfixed** bone marrow trephine is also suitable. Fluid samples such as CSF, ascitic fluid, and pleural fluid do not require anticoagulation. Spare tissue for flow cytometry e.g. lymph node excisions or core biopsy tissue should be placed into culture medium containing Preservative Free Heparin (provided by the Cytogenetics unit in Medical Genetics), or saline if this is not available. Alternatively, spare tissue for flow cytometry can be disaggregated at source to provide a cell suspension, but this **must** be sent in culture medium to preserve the cells. The tissue or cell suspension should be sent promptly to Cellular Pathology in the first instance. Fixed tissue, while suitable for histological assessment, **cannot** be used for flow cytometry.

Flow cytometry

Cells in suspension are stained with a variety of monoclonal antibodies, which are conjugated to fluorescent dyes. Staining is performed in a series of tubes containing specific antibody combinations to provide the maximum information about the different cell populations in the sample. The stained cells are analysed by flow cytometry, where laser light is utilised to characterise the physical structure of the cells, and measure their fluorescent staining. Fluorescence is semi-quantitative, and pattern recognition is required to define malignant cells and distinguish them from background normal cells in the sample. This process is relatively quick – preliminary information on cell lineage and maturation can be provided within a few hours of sample receipt, although a comprehensive cell phenotype will take longer.

Specimen containers and dispatching of samples

Labelling for sample containers and request forms must comply with national guidelines. Cardiff and Vale UHB operates a zero tolerance “Right First Time” labelling acceptance policy. The hyperlink to this is:
http://nww.cardiffandvale.wales.nhs.uk/pls/portal/docs/PAGE/NEWS_POST_BOX/NEWS_2016_TO_2021/2019/RFT_POSTER.PDF

All samples being referred from other hospitals should be placed in a suitable, fully labelled, rigid container (such as Hayes DX). To avoid unnecessary delays, Immunophenotyping samples **MUST** be sent in a separate container **directly** to the Immunophenotyping laboratory. Samples requiring FISH or PCR tests must be sent **directly** to Medical Genetics. **NEVER** send Immunophenotyping and Medical Genetics samples together in the same container – there is a real danger of the samples becoming delayed or lost in the system during transfer between laboratories.

All external samples MUST be pre-registered on TRAKCare LIMS, and electronically dispatched using the Send Tests module. Samples must be fully labelled, **and** accompanied by a fully completed request form and packing slip, **both** of which must contain relevant clinical information (see Table 1 below). Note that a request form

MUST be sent even for samples that have been electronically requested for other tests and then subsequently reflexed.

Table 1: Essential requirements for samples, request forms, and packing slips:

Request form	Sample container	Packing slip
Full patient name and DOB	First name and Surname, plus a minimum of one point of identification from the list of three below	Full patient name and DOB
Hospital number and/or NHS number	1. Date of Birth	Full patient address
Patient gender	2. Hospital ID number	Referral reason/clinical details/test required
Full patient address	3. NHS number	TRAKCare LIMS episode barcode
Referral reason/clinical details/test required		Packing slip number
Full treatment details		Sample type
Sample type		Requesting hospital
Consultant name		
Request form		
Signature of requesting clinician		
Date and time of collection		
Address to send report to		
High risk status (if appropriate)		
TRAKCare LIMS episode barcode (electronically readable)		

Important Notes:

1. Clinical details should be as complete as possible, as inadequate information may lead to delay/inappropriate processing of the sample. It is unacceptable to simply request “Cell Markers” or “Immunophenotyping”

2. It is particularly important to provide details of any Immunotherapy that the patient is undergoing e.g. Blinatumomab, Daratumomab. This type of therapy will often affect the flow cytometric staining/detection of important target antigens and change the interpretation of the data.

3. The Cardiff and Vale UHB “Right First Time” Policy discourages the use of consultant initials. If the signature cannot be identified, the report will automatically be sent to the lead Haematology consultant in the appropriate Health Board, leading to possible delays.

4. Any peripheral blood sample referred for investigation must be accompanied by a copy of a full blood count that has been done **on that sample**. Please send the FBC result AND reticulocyte count with samples requiring screening for Hereditary Spherocytosis.

5. Morphological assessment is an important part of the investigative process for acute leukaemia, lymphoproliferative disorders and lymphoma. As samples of blood and bone marrow can take up to 2 days to reach the laboratory we would request that **two labelled unstained smears** are prepared and sent with the sample – this will avoid any EDTA changes to morphological features and aid interpretation when signing out the report.

6. The samples should be fresh and transported to the laboratory with the minimum of delay. Most samples will remain viable for about 3 days if stored at room temperature. However, this is extremely variable and depends on the sample type, and the nature of the malignant cells in the sample. Labile samples such as CSF or samples containing high-grade disease (particularly Burkitt Lymphoma) will deteriorate more quickly and **will require taxi transport,** rather than hospital courier. Store CSF or serous fluid samples in the fridge if they cannot be dispatched the same day**. Please** contact the laboratory in advance when sending these samples, or if samples require **urgent processing.**

7. Samples requiring interim results on the same day MUST be received **in** the Immunophenotyping laboratory by 12:00 pm. Samples received after 12:00 pm on Friday afternoon MAY not be processed, as the laboratory is particularly busy at this time.
   N.B. Separate restrictions apply for Hereditary Spherocytosis screens; refer to section on HS screening.

Policy for Processing Unlabelled Samples

It is general Cardiff & Vale University Health Board policy that unlabelled samples CANNOT be processed. However, many samples sent for Immunophenotyping are of a "precious" nature - these are frequently obtained by means of an invasive procedure, and the re-taking of the sample would either subject the patient to an additional procedure, or re-sampling may not be possible (e.g. lymph node excision). Therefore, unlabelled samples of a precious nature *will* be processed but **no** results will be released under **any** circumstances until the following criteria have been met:

1. Cardiff and Vale UHB samples: the sample must be labelled by the clinician who took the sample; or if he/she is unavailable, by a clinical colleague willing to accept responsibility for labelling. The clinician labelling the sample must also sign a disclaimer statement.

2. External samples:

   a. Part A of page 2 of the disclaimer form (LF-HAE-IPUnlabelledIndemnity) will be completed by a senior staff member of the Immunophenotyping laboratory, and the whole form scanned and emailed to the source department for the attention of the requesting clinician or a senior managerial colleague.
   b. Part 2 of the form must be fully completed, signed, and sent back by post or internal mail to the Immunophenotyping laboratory.
   c. Where time is of the essence a preliminary verbal report may be provided, but only **after** return by email of a scanned copy of the completed indemnity form to the Immunophenotyping laboratory. The original form **must** still be returned to the Immunophenotyping laboratory before the final report is issued electronically or by hardcopy.

Policy for Processing High Risk Samples

All samples and request forms from known high risk patients must be **clearly labelled** as such, preferably by the use of yellow infection risk stickers. For most investigations, samples that are definitely or potentially positive for HIV, Hepatitis B, Hepatitis C, or TB, can be processed using modified protocols, reducing the infection risk to laboratory staff. However, it is impossible to perform some tests (e.g. cytospins and screening for Hereditary Spherocytosis) on high risk samples.

Due to the possibility of CJD, all CSF samples processed from Neurology are automatically treated as high risk and must undergo a clinical vetting procedure before the sample is taken (see section on CSF samples below).

Testing and Retention of Patient material Policy

By submitting a sample the referring clinician confirms that appropriate consent has been obtained for the testing, use and storage of patient material. It is also assumed that consent is given to send material to appropriate clinical trial laboratories.

The Immunophenotyping laboratory stores stained slides prepared from patient samples for 2-3 years, unstained smears are discarded approximately fortnightly. Samples are disposed of on a weekly basis.

Prioritisation of Samples

URGENT

All patients with suspected acute leukaemia, high-grade lymphoma, or CNS disease, will be treated as urgent and the samples analysed on the day of arrival whenever possible. A verbal (or email) communication of the results will be made to the requesting clinician. Electronic reports will be authorised as soon as possible (see individual test sections below), but may take longer if additional tests (such as FISH) are required. Requests for the dye-binding screen for Hereditary Spherocytosis are also prioritised because of possible degradation of the sample.

ROUTINE

All other samples are treated as non-urgent and analysed as soon as possible.

Samples Stored Awaiting Confirmation of Diagnosis

Non-urgent samples with an uncertain diagnosis or a diagnosis of Myelodysplastic Syndrome (MDS) or Myeloproliferative Neoplasm (MPN) will be stored pending morphological assessment. **These samples will ONLY be processed if there is a communication to proceed from the requesting clinician or from a Haematology clinician at C&V UHB, or if there is a clear indication on the request form.** This avoids unnecessary, costly, labour-intensive analysis being performed on patient samples where a haematological neoplasm has not been confirmed, or where flow cytometry will not be helpful in establishing the diagnosis. Samples will deteriorate with time, so **prompt** communication with the laboratory is essential.

Samples **not** processed by the laboratory

The laboratory does not offer a validated test for the assessment of platelet glycoproteins. Samples requiring this investigation must be sent DIRECTLY to Bristol Royal Infirmary (see section below for further details). The Cardiff Immunophenotyping Laboratory is not responsible for forwarding such samples to BRI.

Flow cytometry is unhelpful in the following conditions:

1. Chronic Myeloid Leukaemia, in chronic phase.
2. Myeloproliferative Neoplasm, unless in transformation.
3. Staging bone marrows for Hodgkin lymphoma.
4. Aplastic anaemia, unless a basic blast count is required.

These samples will NOT automatically be processed unless there is a prompt communication from the referring clinician.

Electronic Reporting Policy

The laboratory aims to provide authorised reports within the declared/published Turnaround Time (TAT). However, this may depend on the cumulative number of individual test(s) requested and the complexity of the investigation for each sample. Any delays in reporting are usually attributable to the non-availability of senior staff, as most flow cytometry reports are complex and may require verification input from more than one senior staff member. Samples of an urgent nature (see above) will always be prioritised for reporting. Authorised reports are available on the Welsh Clinical Portal.

All reports on TRAKCare LIMS are electronic, and the requesting centre is responsible for generating paper copies if required. All new cases of Acute Leukaemia or Lymphoproliferative disorder (LPD) are reported using a Combined Report system. The full report may not be available until 28 days after sample receipt, although it will be possible to view the Immunophenotyping component of the report on LIMS before that time. Clinicians are reminded that any flow cytometry results should be interpreted alongside the results of the other investigations performed on that sample, when they become available.

It is current Cardiff & Vale University Health Board policy that reports CANNOT be sent via FAX.

Enquiries and Requests

Please do not hesitate to contact the Immunophenotyping laboratory to convey relevant clinical information, request provisional results, or prioritise sample processing. Members of staff are happy to co-operate in dealing with such requests, including provision of appropriate references, where available. Email communication is much preferred, as it is less disruptive to the laboratory.

Interpretation/Clinical Advice

If further information is required concerning the interpretation of Immunophenotyping results, or to discuss if sample referral is appropriate, please contact: -

Head of Immunophenotyping Laboratory: Steve Couzens
Tel: 029 20186720, C&V internal extension 46720
Steve.Couzens@wales.nhs.uk

Deputy: Ian Phillips
Tel: 029 20183458, C&V UHB internal extension 43458
Ian.Phillips4@wales.nhs.uk

For clinical advice, please contact:
Dr A.P. Goringe (telephone secretary on 029 2182033, C&V UHB internal extension 42033).

Contingency service arrangements

In the event of catastrophic loss of the service (e.g. due to fire or flood) the following temporary contingency plan will be put into action.

Single platform enumeration:

1. CD34+ cells
2. CD3+ cell (renal transplantation)
3. CD4+ cells

Testing will be carried out by Immunophenotyping staff using the flow cytometers in the Department of Biochemistry and Immunology at UHW. Please send samples to the Haematology Immunophenotyping laboratory as usual.

Leukaemia and lymphoma Immunophenotyping: this service will be provided by the flow cytometry laboratory in the Department of Haematology at Bristol Royal Infirmary. C&V UHB service users should continue to send samples to Haematology Immunophenotyping at UHW – these will be forwarded to BRI. Non C&V service users should send samples **directly** to BRI (address below), as this will minimise any delay in sample testing.

BRI contact details: Ulrika Johansson / Michelle Crawford
Tel: 0117 3422596

Address for samples: Att. Ulrika Johansson
Flow Cytometry Laboratory
Bristol Royal Infirmary
Queen's Building, Level 8
Upper Maudlin Street
BRISTOL
BS2 8HW

Disease Specific Service Provisions

Acute Leukaemia

Samples should be less than 24 hours old, and will be stained with a basic screening panel containing antibodies against CD3, CD7, CD10, CD19, CD15, CD13, CD33, CD34, CD117, HLADR, CD36 and CD45. Peripheral blood samples can be screened in the first instance, and a fuller panel used for the subsequent bone marrow sample which will include important intracellular markers such as TdT, CD79a, myeloperoxidase, cytoplasmic CD3 and lysozyme. Where a bone marrow sample is unlikely to be referred (e.g. an older or frail patient) please inform the laboratory so that a complete phenotype can be determined from the peripheral blood sample. The screening panel will include staining for surface kappa/lambda (to exclude high grade B-cell lymphoma).

As part of a secondary panel, antigens are also tested for their potential use in minimal residual disease testing (see below). These include CD38/CD56/CD123 (AML), CD38/CD123/CD58/CD22 (B-ALL), and CD99/CD48 (T-ALL).

The laboratory no longer performs PML staining for suspected cases of acute promyelocytic leukaemia, as it duplicates information that is provided by gold standard PML/RARA FISH. Bone marrow slides are best for FISH, but peripheral blood slides can be used, provided that enough abnormal cells are present. Slides can be sent direct to the All Wales Regional Genetics Service, or can be forwarded from Immunophenotyping on request.

A verbal or email report will be issued on all new cases of acute leukaemia, and the Immunophenotyping results are available on LIMS or the WCP within 14 days. A full Combined Report (Cardiff and Vale UHB in-patients **only**) is generally produced within 28 days, following MDT discussion.

Blast/Monocyte count

A basic antibody panel is available to detect the numbers of myeloid blasts and monocytes in the sample. An authorised report is available within 28 days.

Measurable Residual Disease

Our laboratory utilises 8-colour flow cytometry to measure the extent of any remaining disease after treatment has started (Measurable Residual Disease, MRD). The sensitivity of this assay depends on the degree of aberrancy of the diagnostic blasts i.e. how phenotypically different the malignant cells are compared to normal bone marrow progenitor cells. Therefore, we establish a comprehensive blast phenotype at diagnosis. For patients not diagnosed at C&V UHB, MRD assessment will still be possible but may not be as sensitive, as the full blast phenotypic profile may not be available, or may be incomplete.

If the diagnostic blasts do not show sufficient aberrancy, other methods for MRD assessment (such as PCR) may be more appropriate/sensitive. This should be evaluated on a case by case basis. Paediatric ALL patients on UKALL2019 are only currently assessed at specific time points: diagnosis, Day 8, Day 28, and relapse. However, Immunophenotyping can be requested at any stage of treatment if the clinician has any concerns.

Samples for MRD assessment should **always** be EDTA bone marrow. Peripheral blood is unsuitable unless the patient has obviously relapsed. It is very important to specify the treatment stage on the request form, as this will greatly help the interpretation of the results. An authorised report is provided within 28 days.

MRD testing by flow cytometry is difficult and time-consuming, and dependent on a number of technical variables. Acquisition of cells on the flow cytometer takes much longer than for other tests, and data analysis/interpretation may be difficult. The sensitivity of the test is 0.1%, and this cut-off is used in the report to make the distinction between MRD positive or MRD negative cases. Samples containing likely <0.1% disease will be reported as MRD Negative. The level of disease cannot be reliably quantitated below 0.1%, but a caveat statement is included in the report.

MRD reporting requires input from two senior members of staff and a full report is available on LIMS within 28 days. We will do our best to accommodate any requests for a provisional report, but requests should be made by email so that Immunophenotyping staff have time to respond in a timely manner. Please note that we are **unable** to provide “on the spot” MRD results either face to face or by telephone – the interpretation requires careful consideration, often by more than one senior member of staff, particularly if the results are not straightforward.

Myelodysplastic Syndrome (MDS)

The role of flow cytometry in the diagnosis of MDS is yet to be fully defined, but several publications have highlighted its usefulness. Diagnosis is made primarily using clinical presentation, morphology, special stains, genetics and Next Generation Sequencing. Our laboratory does not currently provide a full MDS testing flow service, although this could be a potential future development and would ONLY be possible with additional resourcing. We can offer basic testing for absolute numbers of blasts and monocytes with a combination of markers including CD34, CD117, HLADR, CD36, CD64 and CD14. We do not have a validated test to assess dysplasia in the erythroid lineage. An authorised report is available within 28 days.

Systemic Mastocytosis

The laboratory does not have a specific assay for mast cells, but we can detect and enumerate these cells in a standard acute leukaemia or Blast/Monocyte assay using a combination of antibodies, including CD117. More detailed phenotypic characterisation of the cells (e.g. to assess expression of CD2/CD25) is currently not possible – it is best to send a BMA or BMT sample to Cellular Pathology for morphology/Immunohistochemistry.

Lymphoproliferative Disorders

Samples are usually peripheral blood or bone marrow, and should be less than 48 hours old for optimum results (24 hours for serous fluids). Samples suspected of containing high-grade disease should be transported **urgently** to the laboratory, as the cells will deteriorate very quickly. Screening is done with an 8-colour antibody panel to determine the cell lineages present (B-cell, T-cell or NK-cell). B-cell clonality is assessed using reagents directed against kappa and lambda light chains. Samples containing B-cell clones are stained with additional 8-colour tubes to complete the diagnosis. If there are no clonal B-cells present, the investigation may be concluded, unless the following criteria apply:

1. The absolute lymphocyte count is > 7.0 $x10^9$/litre
2. The cells show abnormal morphology, as reported by the requesting laboratory
3. There is a specific request from the clinician
4. There are clinical indications e.g. mediastinal mass
5. The request is for a definite disease e.g. Sezary cells

Samples containing suspected abnormal T-cell populations will be investigated further with an 8-colour panel of additional T-cell and NK-cell markers. T-cell clonality can often be demonstrated by assessment of the TVbeta repertoire. The sample can be sent for PCR if no clonality is demonstrated by flow cytometry. It should be noted that oligoclonal bands can sometimes be detected by T-cell PCR in patients with reactive conditions, or in older patients (>70 years of age). Discussion with Medical Genetics is required before requesting PCR on such samples.

All new cases of LPD or lymphoma identified/discovered by flow cytometry will be discussed at a weekly MDT Meeting, and further work will be considered if necessary. All cases of CLL with a score of <3/5, will receive FISH for the IgH/CCND1 translocation to exclude mantle cell lymphoma, in accordance with BCSH guidelines. Samples suspected of containing follicular lymphoma or DLBCL may receive FISH investigation for IgH/BCL2, BCL6, or MYC as appropriate. CLL samples can be tested for trisomy 12, TP53, ATM and del (13q) by FISH, but this must be requested in advance, or can be done on a subsequent sample preferably sent directly to the Regional Medical Genetics unit.

All new cases of LPD or lymphoma will be reported on LIMS using a Combined Report. The Immunophenotyping component of this report is usually available to view within 14 days, but the full report may take longer, depending on the number of additional confirmatory tests performed by other laboratories. It should be noted that any Immunophenotyping results viewed singly should be correlated with the full report, when it is available.

LPD Measurable Residual Disease (MRD)

The laboratory does **not** offer a validated test to detect MRD in patients with a Lymphoproliferative Disorder, and there is no specific reporting set on LIMS. The standard diagnostic antibody panels may be used, but the sensitivity of this approach may vary depending on the disease phenotype and the amount of residual disease present. An authorised report is available within 28 days.

Plasma cell dyscrasia

Clonal proliferations of plasma cells occur in Monoclonal Gammopathy of Unknown Significance (MGUS), Plasmacytoma, Amyloidosis, Lymphoplasmacytoid Lymphoma, Multiple Myeloma and Plasma Cell Leukaemia. Plasma cells can be quantitated by flow cytometry and their phenotype and clonality established. However, plasma cell numbers are often higher on the bone marrow aspirate film. This apparent disparity may be due to a number of reasons: patchy disease, haemodilute samples, and loss of cells during sample preparation.

Samples are stained with a single 8-colour tube containing antibodies to cytoplasmic kappa/cytoplasmic lambda, CD20, CD19, CD138, CD38, CD56 and CD45. Samples should be less than 48 hours old for best results. An authorised report is usually available within 28 days.

Myeloma Measurable Residual Disease (MRD)

We do **not** currently offer a validated flow cytometry test to measure Myeloma MRD, and other methods should be considered. The diagnostic Myeloma antibody combination listed above may be used, but the sensitivity is probably only 1%, and very dependent on sample/testing variables and the presence of background polyclonal B-cells and plasma cells.

Lymphoma investigations (tissue and serous fluids)

Samples are either solid tissue such as lymph node excisions/core biopsies, or fluids such as Fine Needle Aspirates (FNA) or serous fluids (ascitic fluid, pleural fluid). Fresh samples are essential and fixed material **cannot** be used for flow cytometry. Solid tissue should be placed into culture medium containing Preservative Free Heparin (PFH), or saline if this is not available, and transported **urgently** to Cellular Pathology for disaggregation in the first instance. An aliquot of disaggregated cells, or a single core biopsy, will be transferred to Immunophenotyping for flow cytometry. Speed is of the essence - samples greater than 24 hours old will often show poor viability.

Lymphoma diagnosis is still centred on traditional morphology and immunohistochemistry. Other test results such as flow cytometry, FISH and PCR may be used by the All-Wales Lymphoma Panel (AWLP) to provide an integrated report. Flow cytometric screening is very similar to that performed on LPD samples but the selected panels are designed to detect a wider range of malignancies, including non-haemopoietic tumours. There is also greater emphasis on assessment of Ki-67 proliferation to grade the tumour.

Results for flow cytometry MUST always be interpreted alongside the morphology/cytology/IHC results. Stand-alone flow cytometry reports on LIMS or the WCP will always contain a statement to this effect. The flow cytometry report is authorised on LIMS within 28 days, and may be incorporated into the wider histology report if considered appropriate. Interim results may be communicated, preferably by email.

CSF flow cytometry

Flow cytometry is useful to investigate CSF samples for the presence of lymphoma or acute leukaemia. **At least** 5 ml of sample is required and the sample must be tested as quickly as possible due to the likelihood of rapid cell deterioration. The sample should be stored at 4^0C if it cannot be tested the same day. Cytospins should be made in order to provide morphological correlation with flow cytometry, unless the samples have originated from Neurology (see below).

Requests for CSF flow cytometry originating from Neurology must firstly be vetted by one of the Haematology clinicians, to determine if they are clinically justified, as the incidence of true CNS lymphoma in this patient group is extremely low. The risk of CJD must also be ascertained – if CJD is included in the differential then the sample **must not** be processed unless the patient has been appropriately tested. Cytospins are **not** made on these samples. If morphological correlation is required, a **separate** sample should be sent direct to Cytology.

The results from CSF flow cytometry testing are authorised within 3 days.

Non-Haematological cancer

Flow cytometry can also be used to **screen** for non-haematological cells in BMA samples. The antibody EpCAM (CD326) stains epithelial cells brightly, and a normal BMA sample should not contain any such cells, although weak EpCAM staining may be seen on nucleated red cells. CD56 positivity may assist in the diagnosis of small cell carcinoma (EpCAM+/CD45-/CD56+), or neuroblastoma (EpCAM-/CD45-/CD56+). However, this test has not been fully validated and will only be offered as a UKAS unaccredited test.

The flow cytometry authorised report is available within 28 days.

Cell Enumeration

Flow cytometry is used to accurately count specific cell populations in a number of different samples and therapeutic products. Cells are quantitated using monoclonal antibodies in a single platform technique. A calibrated suspension of fluorescent microbeads provides a reference source and the concentration of cells is determined by the ratio of the cells to the beads, multiplied by the bead concentration.

The following cell populations can be measured by this method:

1. CD4+ and CD8+ T-cell subsets: for Haematology patients post-BM/PBSC transplant or on treatment

2. CD34+ stem cells and/or CD3+ T-cells in peripheral blood, autologous/allogeneic PBSC harvest or Bone Marrow products. For allogeneic assays, the viability of the cells is also assessed using the nuclear dye 7-AAD. Results may be communicated directly to staff in the Stem Cell Processing Unit (SCPU), but never to any other staff: clinical/nursing staff must contact the SCPU for any advice about testing or results.

3. CD3+ T-cell enumeration in peripheral blood from patients undergoing solid organ transplant and receiving OKT3 or ATG therapy. This service is only provided to Renal Medicine **within** Cardiff and Vale UHB.

Requests for the following should be sent directly to the Medical Biochemistry and Immunology Department at C&V UHB, and **NOT** to Immunophenotyping:

a. CD4+ and CD8+ T-cells subsets for all Non-Haematology patients with primary or acquired immunodeficiency (GUM patients, CVID, ALPS, Di-Georges syndrome).

b. Estimation of total B cell numbers in patients receiving B cell immunotherapy (e.g. Rituximab) for Rheumatoid Arthritis or Multiple Sclerosis.

c. Requests for patients receiving Campath immunotherapy.

Samples used for testing are fresh peripheral blood in EDTA, apheresis products or bone marrow harvests; results for peripheral blood CD34 tests are authorised within 2 hours of sample receipt, as this will guide the decision to proceed to apheresis. The results from allogeneic CD34 and/or CD3 testing are reported within 3 hours. Renal CD3 tests and CD4 tests are authorised within 6 hours and 7 days, respectively.

Hereditary Spherocytosis (HS) screening

The laboratory can employ an EMA dye-binding test to screen for Hereditary Spherocytosis (HS); however, this test is labour intensive, very time consuming and requires the use of sample time-matched controls. As such, the laboratory **MUST** be contacted well in advance **before** making arrangements to bleed the patient, so as to ensure adequate staff availability to perform the test.

A pre-transfusion sample is **essential.** Samples can only be processed at the beginning of the week (Mon/Tue/Wed) and **cannot** be accommodated on Thursday or Friday. On Wednesdays, samples **must** be received early to ensure processing that day; a **stringent** latest cut-off time for receipt by 1:00 pm applies. It is the responsibility of the dispatching laboratory to ensure that the sample arrives *in the Immunophenotyping laboratory* in time to be processed; dispatch by taxi may be preferable, and the transportation package must be labelled as URGENT. On occasions, due to workload demands and staff availability, we may not be able to offer the test.

The test should be considered as one of "last resort", **only** to be requested in patients with an unexplained haemolytic anaemia where an immune cause for the haemolysis, and also common enzymopathies such as G6PD deficiency, has been previously excluded. In addition, in those cases where an immune mechanism, or enzymopathy, have been excluded, the BCSH guidelines on Diagnosis and Management of HS (2011) asserts that the use of a screening test (e.g. EMA binding) to establish a diagnosis of HS is **not warranted** where there is a pre-existing family history of HS allied to classical presentation features.

The assay is principally only a screening test for HS, and is not intended to provide a definitive diagnosis; family history, clinical presentation and FBC/reticulocyte count and other tests **must** be considered before requesting the test, and then taken into account before making the final diagnosis. A copy of the FBC and reticulocyte count MUST be included with the sample.

Patients with HS show a 20-30% decrease in red cell dye binding fluorescence compared to normal controls. The test shows >99% specificity and >92.7% sensitivity for HS, but does not exclude some Band 4.2 and ankyrin deficiencies. Decreased dye binding is also seen in Southeast Asian Ovalocytosis, Hereditary Pyropoikilocytosis and Cryohydrocytosis, but results are not as clear-cut and the test is most useful for HS screening. A definitive diagnosis may require Next Generation Sequencing technology.

An authorised report is available within 28 days.

Paroxysmal Nocturnal Haemogobinuria (PNH)

GPI-deficient red cells and granulocytes can be reliably detected by flow cytometry. De-novo PNH is very rare, but small clones can emerge in patients with aplastic anaemia and MDS, suggesting disease evolution. The laboratory measures CD55 and CD59 on red cells, and Type I (normal), Type II (partially deficient) and Type III (fully deficient) red cells can be quantitated. Granulocyte expression is a qualitative assessment via measurement of FLAER, CD24, and CD16. Monocytes are not currently assessed.

PNH testing should only be considered in specific patient groups because of the low incidence of GPI-deficient clones. Please refer to the Wales PNH referral guidelines on the C&V UHB Haematology services Intranet page:

http://nww.cardiffandvale.wales.nhs.uk/portal/page?_pageid=253,972276,253_972286&_dad=portal&_schema=PORTAL

In particular, PNH testing as part of a **general** Thrombophilia screen should be discouraged, unless there are accompanying clinical indicators such as haemolysis, aplasia or **very unusual** sites of thrombosis (please state the site of thrombosis on the request form).
The sample should be fresh EDTA peripheral blood. Bone marrow should **never** be tested as expression of some GPI-linked antigens (CD16) is maturation dependent. A pre-transfusion sample is essential, as any red cell clones will be diluted by the

transfused cells, leading to underestimation of the clone size. The granulocyte clone is unaffected by transfusion because blood products are leuco-depleted. Samples older than 24 hours will be more difficult to interpret. Neutropenic samples are also problematic.

An authorised report is generated within 28 days of sample receipt.

Platelet Glycoproteins

Testing for platelet glycoproteins is no longer provided by our laboratory. Samples can be referred to the flow cytometry laboratory at Bristol Royal Infirmary; however, please contact BRI **in advance** by telephone to discuss testing and transportation arrangements. Samples must be sent direct to BRI and **not** via Immunophenotyping UHW. The test requires citrated blood, and a control sample is required. Please discuss details with the laboratory prior to sending samples.

BRI contact details: Ulrika Johansson / Michelle Crawford
Tel: 0117 3422596

Address for samples: Att. Ulrika Johansson
Flow Cytometry Laboratory
Bristol Royal Infirmary
Queen's Building, Level 8
Upper Maudlin Street
BRISTOL
BS2 8HW

Immunoplatelet counting

This test is useful when impedance analysers used for routine Haematology full blood counts are unable to accurately define and count the platelet population, due to the presence of small or abnormal red cells in the sample, or large platelets. Platelets are accurately counted by flow cytometry using a fluorescent antibody against CD41. The platelet count is calculated from the ratio of the red cell events to platelet events, then multiplied by the red cell count from the Haematology analyser and scaled up by a factor of 1000 to take into account the relative frequencies of the platelets and red cells.

The sample should be EDTA blood, within 24 hours of collection. As the red cell count is used in the calculation of the Immunoplatelet result, the reporting set on TRAK LIMS is configured to “pull” the RBC data from the FBC on the same laboratory episode number, so it is **vital** that an FBC is performed *first* on the sample **before** referring it to Immunophenotyping. The test results are usually available on LIMS within 6 hours.

Foeto-maternal haemorrhage (FMH)

Foetal red cells present in the maternal blood circulation as a result of feoto-maternal haemorrhage can be detected by flow cytometry. The test provides a more precise estimate of bleed volume than is otherwise obtainable with the standard acid elution (Kleihauer) test; the flow cytometry method is more sensitive and less subjective.

Flow cytometry is used to detect RhD+ foetal cells in RhD-ve mothers, and quantitates the bleed volume to ensure accurate prophylaxis. This method **can** be performed on samples of known infection risk, provided that additional precautions are taken.

RhD FMH testing requires a sample of EDTA blood, as fresh as possible. For delivered babies, the Rhesus groups of the mother and baby must be supplied on the request form, along with the date and time of delivery. Testing should take place **BEFORE** anti-D prophylaxis. The blood transfusion laboratory is given a verbal report as soon as the flow cytometry results are known; an authorised report is available on LIMS within 24 hours of testing. The Immunophenotyping laboratory **does not** provide advice on Anti D prophylaxis – please contact the Transfusion Laboratory.

Other FMH flow cytometry methods may be helpful if the mother is RhD+, or if the RhD group of the baby is unknown. Testing is based on the detection of high levels of HbF in foetal red cells compared to adult F cells (kleihauer “intermediates”) or normal adult red cells, using a combination of antibodies against both HbF and carbonic anhydrase. **These additional FMH tests are not available in our laboratory, and this service is only offered by the Welsh Blood Service (contact UHW BTL initially)**.

Cytospins

Cytospins are prepared primarily from CSF samples and offer an opportunity for morphological cell assessment to complement flow cytometry, or to justify its use.

The samples need to be as fresh as possible. Cytospins will **not** be made on samples with an infection risk (flow cytometry will be performed instead):

1. Known high risk patients
2. Serous fluids (due to the risk of Covid-19)
3. Samples from patients on the Neurology or infectious disease wards.

Morphology is often reported in conjunction with flow cytometry, and the authorised report is usually available within 3 days.

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