TECHNICAL NOTE

Flow cytometry crossmatch (FCXM) with the XMStep kit – T/B identification, clinical interpretation and controls

1. Identification and separation of T and B lymphocytes (gating and population percentage)

The e The Immunostep **XMStep** kit is designed to detect preformed anti-HLA antibodies in recipient serum against donor lymphocytes by flow cytometry[1][2]. Under the recommended conditions, donor **mononuclear lymphocytes** (including both T and B lymphocytes) are first **isolated** by density gradient (e.g., Ficoll) or another equivalent method[3]. This provides a lymphocyte-enriched cell suspension (typically with a majority of T cells ~70% and a minority of B cells ~5-15%, depending on the donor). These cells are then incubated with the recipient's serum and with positive and negative controls, according to the kit instructions (200 µL of cell suspension with 40 µL of serum in each tube). After washing to remove unbound antibodies, the kit reagent (a FITC-conjugated anti-human IgG monoclonal antibody) is added to reveal the presence of IgG bound to the cell surface[5]. Finally, the cells are analysed in the flow cytometer.

Differentiation of T vs. B lymphocytes: Since the preparation contains both cell types, it is essential to differentiate them during acquisition and flow cytometric analysis. The kit manual specifies that the appropriate **acquisition settings and gates** must be defined by the user according to the flow cytometer used[6]. In practice, there are two main strategies for achieving separation of T and B populations:

- **a)** Multicolour **gating**: This is the strategy recommended by technical guidelines. It consists of adding monoclonal antibodies labelled against **CD3 (pan-T)** and **CD19 or CD20 (pan-B)** to the assay in order to simultaneously identify T and B lymphocytes during analysis[7][8]. For example, CD3 can be labelled with PE or APC to delineate T lymphocytes, and CD19/CD20 can be labelled with another fluorochrome (e.g. APC or PE-Cy5) to label B lymphocytes. Thus, after acquiring the data, a gate is set in the lymphocyte region (based on forward/side scatter) and then a gate is applied in the channel corresponding to CD19/CD3 to separate the subpopulations: the **CD3+ (T lymphocytes)** and **CD19+ (B lymphocytes)** populations are clearly defined[7]. This multicolour approach is the most robust and is supported by the literature, as it allows the reactivity of anti-HLA antibodies to be measured **specifically in T and B cells** within the same sample, improving the specificity of flow crossmatching[7].

- **b) Physical separation of subpopulations:** Some laboratories choose to physically isolate donor T and B cells prior to testing (e.g., using immunomagnetic methods or E rosettes). With this strategy, two separate cell suspensions are prepared: one enriched in T lymphocytes and the other in B lymphocytes. Each fraction is separately subjected to the incubation protocol with sera (negative control, positive control and recipient serum) and labelling with anti-IgG-FITC[9][10]. Finally, the tubes corresponding to **T crossmatch** and **B crossmatch** are analysed separately in the cytometer. This methodology, although requiring additional steps, ensures that more than 95% of the cells in each tube are from the population of interest, thus facilitating interpretation (e.g., eliminating the contribution of B cells to T crossmatch, and vice versa).

In both approaches, it is important to verify the **purity or percentage of separation** achieved: ideally, the identified B population should represent the expected percentage of B lymphocytes in peripheral blood (e.g. ~5-15%) without significant T contamination, and vice versa for the T population (~70-80% of lymphocytes)[8]. Proper gating is evidenced by the two populations forming **well-differentiated clouds or histograms** in the cytometric analysis, without excessive overlap. The XMStep kit leaves the definition of these gates to the user, but its technical data sheet shows examples of histograms where the clear separation of B and T lymphocytes and their different reactivity to control sera can be seen[11][12].

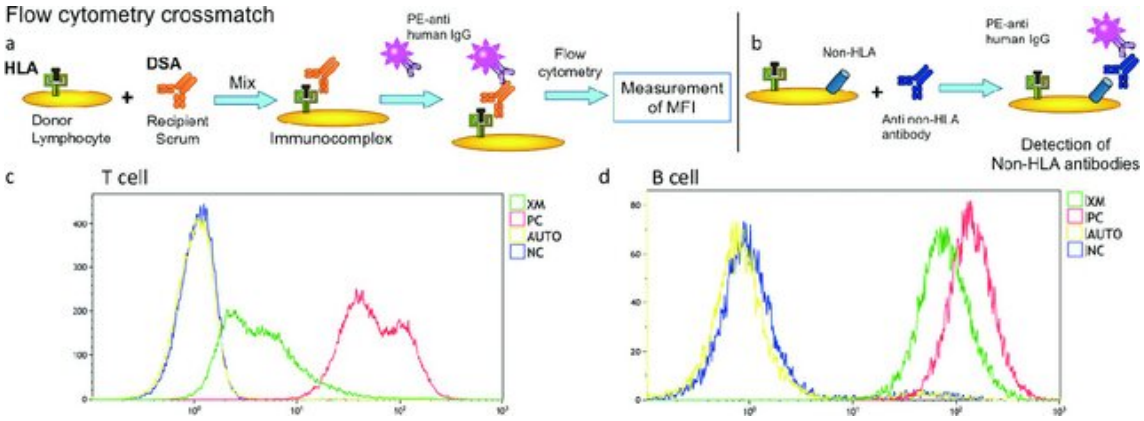

Figure 1. **Diagram and identification of T vs B populations in flow crossmatching.** (a) FCXM assay diagram: donor lymphocytes (with class I HLA antigens in T and class I+II in B) are incubated with recipient serum; if there are anti-donor antibodies (DSA), these bind to the cells. Anti-IgG-FITC is then added, which binds to the attached antibodies, allowing fluorescence to be detected in the target cells[7]. (c) Typical histogram of **T** lymphocytes: the negative control (NC, black/blue line) defines the baseline fluorescence; a positive test serum (XM, green line) shows a shift to the right (increased fluorescence) if anti-HLA class I antibodies are present; the positive control (PC, red line) with known antibodies produces a clear increase in fluorescence. (d) Typical histogram of **B** lymphocytes: since B cells express HLA class I and II, they usually show greater antibody binding; in this example, the positive control serum (pink line) generates a very marked fluorescent shift in B, while the recipient serum (XM, green line) is close to the negative control (blue line), indicating the absence of significant antibodies in this case. *(Image adapted from Nakamura et al. 2018, CC BY 3.0 licence)*[13][8].

In summary, under optimal conditions, the kit allows **quantitative differentiation of T and B populations** through careful gating or prior separation, ensuring that we can evaluate antibody binding in each cell type separately. It is essential to follow the recommendations for cytometer calibration and voltage/compensation adjustment prior to acquisition, as accurate results depend on correct alignment and gating[14][15]. A good indicator of successful separation is that the final histograms/results show a clear distinction between T and B lymphocyte fluorescence, as shown in the examples provided in the kit documentation[11][12].

2. Interpretation of results and clinical report

General criteria: In the interpretation of flow cytometry crossmatching (FCXM), the fluorescence signals obtained with the **recipient's serum** are compared with those obtained with the **negative control serum**. The median channel shift (MCS) or the **increase in the mean/median fluorescent intensity (MFI)** of donor cells incubated with the patient's serum is usually evaluated in relation to the negative control[16]. Each laboratory must establish its own validated cut-offs, ideally based on control distributions and correlation with clinical results[17].

For example, the XMStep kit technical data sheet mentions as **a guideline criterion** that a result could be considered *positive* when the mean fluorescence of cells with the recipient's serum ( ) exceeds **three times** that obtained with the negative control serum[18]. Other laboratories express the result as an **index or ratio** between the median MFI with patient serum and the median with negative serum[19]. In clinical practice, FCXM results are usually categorised and reported as **"negative", "doubtful or weakly positive" or "positive"**, depending on the degree of fluorescence shift relative to the negative control[16].

Reporting results: When issuing the report, **the results for T lymphocytes and B lymphocytes** should be **specified separately**, as each has different implications. For example, it is usually reported in a textual format such as: *"Flow cytometry crossmatch: T cells: negative (MCS= +5 channels), B cells: weakly positive (MCS= +40 channels)"*, accompanied by the clinical interpretation. It is important to include the controls used (negative and positive) in the report, as well as any factors that may have affected the test (e.g., serum dilution, special cell treatments).

The guidelines recommend correlating the reactivity observed in the crossmatch with the presence of **specific anti-HLA antibodies** identified by other techniques (e.g., simple antigen assay in Luminex). In fact, the FCXM result is currently often interpreted in conjunction with the recipient's antibody profile: a concordance between a positive FCXM and the detection of Donor-Specific Antibodies (DSA) against donor HLA antigens in Luminex tests reinforces the conclusion of immunological incompatibility, while discordant results require further analysis[21].

Clinical significance: In the context of solid organ transplantation (classically kidney transplantation), the classic rule of thumb is that a **positive crossmatch** is associated with a higher risk of antibody-mediated rejection, while a **negative crossmatch** indicates the absence of circulating cytotoxic antibodies against the donor and is usually considered a prerequisite for proceeding with transplantation[22].

The following table summarises the typical interpretation of the different FCXM result patterns:

FCXM result (anti-donor IgG)	**Immunological interpretation**	**Clinical relevance**
T positive / B positive	Indicates the presence of recipient antibodies against donor HLA class I antigens (expressed on T and B cells) and possibly class II antigens (expressed only on B cells). Equivalent to DSA against class I and/or II antigens.	High risk of **immediate** rejection. Generally **incompatible**; traditionally contraindicates transplantation[22] unless aggressive desensitisation therapies are applied.
T negative / B positive	Suggests recipient antibodies that bind to antigens present on B lymphocytes but not on T lymphocytes. Typically corresponds to **DSA against class II HLA** (e.g., anti-DR/DQ from the donor), as T cells do not express these antigens. *(Note: in rare cases, it could be due to non-HLA antibodies that specifically affect B cells, or to differences in technical sensitivity.)*	It indicates a risk of mainly **humoral antibody-mediated rejection** (AMR) **(class II)**. Historically, an isolated B+ crossmatch has been associated with early post-transplant rejection[23], which is why many centres consider it **a relative incompatibility**. Transplantation may be considered with caution (e.g. enhanced immunosuppression) if there is no alternative, or prior desensitisation.
T positive / B negative	This pattern *is not expected* for known anti-HLA antibodies, as any antibody against HLA class I would also have reacted with B. Therefore, it usually indicates **non-specific or non-HLA** reactivity. Examples: autoantibodies directed against T lymphocytes (or their Fc receptors), antigen-specific antibodies other than HLA present in serum, or technical interference.	**Careful interpretation**: The presence of identified anti-HLA antibodies in the recipient must be confirmed. If no known DSAs are detected (e.g., in Luminex) and only T is positive, the result may be a **false positive** due to non-HLA antibodies or technical factors[24]. In a clinical context, this isolated result without concurrent DSA may not absolutely contraindicate transplantation, but it warrants further investigation (e.g., repeat testing, evaluate autoantibodies, consider pronase to eliminate B Fcγ receptors, etc.).
T negative / B negative	No significant binding of IgG from the recipient to the donor's T or B lymphocytes is detected, i.e., **there are no anti-donor antibodies detectable** by the assay.	**NEGATIVE** result: suggests acceptable immunological compatibility. In clinical terms, a negative FCXM IgG crossmatch indicates that it is safe to proceed with transplantation in terms of immediate risk of hyperacute rejection by antibodies[22] (always considering other immunological factors).

Table 1. Interpretation of crossmatch patterns by flow cytometry (IgG). It should be noted that the clinical decision is not based solely on these qualitative results, but also on the quantitative intensity of the signal, the correlation with antibody tests (e.g. DSA detected by Luminex) and the patient's context (degree of prior sensitisation, type of organ to be transplanted, urgency, etc.). For example, a **weakly positive FCXM** (close to the cut-off point) could be considered acceptable in certain cases if the antibodies involved are low affinity or non-complement-fixing, whereas a **strongly positive** result usually contraindicates transplantation unless desensitisation is achieved.

Additional recommendations for interpretation: It is essential to always use **controls** to validate each experiment: the **negative control** (serum without anti-HLA antibodies) should give a low and stable baseline fluorescence, while the **positive control** (serum with known anti-HLA antibodies) should show a clear shift towards higher intensities. If the controls do not behave as expected (e.g., the positive control does not stain, or the negative control shows high fluorescence), the results of the patient samples **should not be reported** until the problem is resolved, as the test would not be reliable. It is also recommended to include in the interpretation any circumstances that may affect the result: for example, it is known that the presence of **therapeutic agents such as rituximab** in the donor or recipient can interfere with the assay. ttached to donor B lymphocytes can cause a false positive in B (due to detection of anti-IgG against the attached rituximab) [25]; in such cases (e.g., donor recently treated with rituximab rituximab), a B+ crossmatch may not reflect a real DSA but rather the presence of that drug, which should be considered in the report. Other treatments such as antithymocyte globulin (ATG) or high doses of IVIg in the recipient may also produce non-specific signals[25][26]. Therefore, guidelines (e.g., ASHI) suggest always correlating a positive FCXM with the **search for specific antibodies in Luminex** and with the patient's history, to discern between true anti-HLA antibodies and possible causes of false reactivity[21]. Finally, the report should be written in clear but technical language, stating the main conclusion (*"positive/negative crossmatch"*), accompanied by an interpretation to guide the clinician (e.g., *"indicative of donor-specific class II antibodies; high risk of humoral rejection"*). This ensures that the FCXM result is used appropriately in clinical decision-making.

3. Clinical support and references on the application of FCXM

The use of flow cytometry crossmatching in transplantation is **widely supported by the scientific literature and specialised guidelines**. Since its introduction in the late 1980s (Bray et al., Transplantation 1989, double-colour T/B method), multiple studies have shown that FCXM has greater sensitivity than traditional complement-dependent cytotoxicity (CDC) and provides clinical value in predicting rejection. For example, FCXM is approximately **10 to 100 times more sensitive** than the CDC test in detecting antibodies[27], revealing non-complement-fixing or low-titre antibodies that CDC would miss[28]. Thanks to this high sensitivity, the use of FCXM in the early 1990s showed that subtle immunological incompatibilities associated with accelerated rejection or early graft failure that were previously undetectable could be identified[29]. Pioneering studies such as *Iwaki et al., 1991 (Nephron 57:268-72)* and *Stefoni et al., 1991 (Nephron 57:268-72)* validated the clinical utility of FCXM in renal transplantation[30], finding a correlation between a positive flow crossmatch and poorer graft outcomes. More recently, in the era of single antigen testing (Luminex SAB), it has been documented that a **positive FCXM** remains prognostic: for example, *Couzi et al., 2011 (Transplantation 91(8): in press)* reported that transplant patients with a positive FCXM had significantly higher rates of acute rejection compared to patients with a negative FCXM[31]. These findings confirm that the presence of DSA detectable by FCXM has real clinical relevance.

Professional and regulatory bodies in immunogenetics and transplantation also **endorse and recommend** the FCXM technique. The **American Society for Histocompatibility and Immunogenetics (ASHI)** includes flow crossmatching among the acceptable methods for pre-transplant evaluation in its standards, emphasising that the most sensitive technique available should be used to detect relevant antibodies[32]. In fact, the standards require laboratories to define cut-off policies based on clinical considerations for the most sensitive crossmatch techniques (such as flow)[32], and that there be a **final crossmatch before transplantation** (physical or virtual) for organs such as the kidney, except in emergency circumstances[33][34]. Likewise, international guidelines (such as those of the EFI in Europe and national associations) recognise FCXM as the reference method for detecting preformed antibodies. For example, an Australian guideline on HLA cytometry highlights the multicolour approach of FCXM with CD3/CD19 and anti-IgG, and its ability to identify clinically relevant antibodies that CDC does not detect[28][7].

In terms of **technical protocols**, there are numerous publications and manuals describing the execution and interpretation of FCXM in a clinical setting. For example:

- The *ASHI Laboratory Manual* (latest editions) devotes chapters to flow crossmatching, providing practical recommendations on controls, gating, and cut-off point calculations.
- Review articles such as *Jaramillo et al., "Technical Aspects of Crossmatching in Transplantation," Clin Lab Med 2018* summarise the critical steps of FCXM and discuss interferences and technical improvements[35].
- *Liwski et al., 2018 (Hum. Immunol. 79:28-38)* describe optimised protocols ("Halifax protocol") for performing rapid and reproducible FCXM[36].
- Specific studies, such as *Delgado & Eckels 2008 (Exp. Mol. Pathol. 85:59-63)*, analysed the implication of a **B-only positive crossmatch** in kidney transplantation, showing that such cases (isolated class II DSAs) may be associated with post-transplant humoral rejection if no measures are taken[37].

Taken together, all this evidence and guidelines support the clinical application of FCXM. Today, most kidney transplant programmes (and other organ transplant programmes in sensitised patients) routinely use flow crossmatching as a complement to HLA typing and antibody identification. A typical procedure would be: first, a *"virtual crossmatch"* is performed based on the antibodies detected by Luminex; if there are no apparent DSAs, this is confirmed with a **flow crossmatch** prior to transplantation to detect any unexpected or low-affinity antibodies[38][39]. This combined approach maximises safety, minimising both **false negatives** (which could lead to hyperacute rejection if less sensitive methods were relied upon alone) and **false positives** (e.g. antibodies against low-expression HLA antigens that signal on Luminex but do not damage the graft; a negative FCXM would help to interpret these as likely clinically irrelevant).

In summary, flow cytometry crossmatching is strongly supported by clinical experience and the literature. Its incorporation into the histocompatibility laboratory has reduced the incidence of hyperacute rejection and improved donor-recipient matching by detecting pathogenic antibodies that previous techniques could overlook[28]. Good practice guidelines (ASHI, EFI, etc.) consider it standard practice, provided it is performed with the appropriate controls and within a comprehensive diagnostic algorithm. For any centre considering purchasing the XMStep kit, it is reassuring to know that the methodology it will implement is backed by decades of successful use and studies demonstrating its benefit in graft survival.

4. Positive and negative control sera: recommendations for use

The use of **negative and positive control serum** is mandatory to ensure the reliability of each flow crossmatch test. The XMStep kit makes this explicit: among the "necessary materials not provided" are listed a **negative control serum** and a **positive control serum**, in addition to the recipient's serum[40]. Recommendations for their selection and use are detailed below, including examples of recognised commercial sources:

- **Negative Control Serum:** This must be human serum that **does not contain** relevant **anti-HLA antibodies**. Traditionally, **AB** serum from a healthy male donor who has never been transfused or transplanted (and preferably nulliparous if female) is used to minimise the likelihood of preformed anti-HLA antibodies. A common alternative is to use a **pool of AB sera** from several non-sensitised donors, which averages out any low- r non-specific reactivity. There are also commercially available preparations validated for this purpose; for example, some laboratories use negative sera certified by reference banks. A recognised source is the **NIBSC (National Institute for Biological Standards and Control)** in the United Kingdom, which provides **international reference reagents** for FCXM, including **anti-HLA antibody-negative** plasma/serum[41][42]. European guidelines indicate that the negative control can be an AB pool or a commercial preparation such as that from the NIBSC[43]. In any case, it is recommended to check each new batch of negative serum by verifying that it does not give a signal in antibody tests (e.g., Luminex soluble antigen test)[44]. An optimal negative control serum should produce a narrow fluorescence histogram, overlapping with the autofluorescent background noise of the cells (thus defining the **baseline** against which we will measure shifts)[17]. If the negative control shows elevated or atypical fluorescence, this could indicate the presence of non-specific antibodies or autoantibodies in that serum, and **it should not be used**.

- **Positive Control Serum:** Its function is to ensure that the assay can detect anti-donor antibodies when they are present. Therefore, it must be a serum **with strong anti-HLA antibodies and preferably broad-spectrum** (multi-specific). Ideally, a **pool of sera from highly sensitised patients** (with high PRA, antibodies against multiple HLA class I and II antigens) should be used [43]. By combining several hyperimmune sera, the probability that the positive control will contain antibodies against *any* HLA antigen present in any given donor is increased, ensuring a positive crossmatch reaction. This prevents false negatives in the positive control due to the lack of target antigens in the donor cells. As a reference, the literature describes the use of **"PCS" (Positive Control Serum)** made from a pool of sera from sensitised patients, diluted to an optimal level so that it does not saturate the detection but clearly exceeds the negative signal[45]. Commercial products are also available: for example, One Lambda (Thermo Fisher) offers class I and class II positive control sera (FlowPRA® Positive Control Serum) that are mixtures of sera with known antibodies[46]. These commercial sera are typically validated to give a consistent positive signal in flow tests. Another option is the aforementioned **NIBSC**, which has a **strong** international **positive** reference **serum/plasma** for FCXM (e.g. code **17/212 or 21/378**, plasma pool with broad anti-HLA reactivity)[42]. When using commercial positive serum, it is good practice to verify that it effectively marks the cells of the donor in question; in some laboratories, this is also checked using antibody panel tests covering the relevant antigens. If the positive control is **too potent** (e.g. a hyperimmune serum with a very high titre), it can saturate the fluorescence signal of all B and T cells, making quantitative interpretation difficult; in such cases, dilutions can be tested to adjust the intensity to a useful

dynamic range[47]. The important thing is that the positive control produces a **clear shift in fluorescence** relative to the negative control in both T and B populations[12], serving as a reference for an unequivocally positive reaction.

Storage and handling: Both negative and positive serum must come from *stable* batches. It is recommended to aliquot them into small portions to avoid repeated freezing/thawing that can degrade complement or immunoglobulins. They should be stored according to the supplier's instructions or in a freezer at -70°C for long-term storage, minimising contamination. Homogenise well before use. Some laboratories also include an **auto-crossmatch control** (recipient serum against their own cells) to detect autoantibodies or background noise from the patient's serum; although not mandatory, this can help interpret doubtful results (e.g., a patient with autotropic antibodies could test non-specifically positive with both the donor and themselves).

Recommended brands/summary: In summary, for a laboratory implementing the XMStep kit, the following are suggested:

- **Negative Control:** Reference AB serum (e.g., *One Lambda FlowPRA® Negative Control Serum*, or verified negative AB serum pool). For example, the One Lambda insert describes its negative serum as containing no detectable HLA antibodies and establishes the fluorescence baseline[46]. Alternatively, *NIBSC Anti-HLA Negative Serum, Ref. 17/238*, an international standard, can be used as a negative control.

- **Positive Control:** Pool of sensitised individuals or validated commercial serum with anti-HLA antibodies. For example, *One Lambda FlowPRA® Class I Positive Control Serum* and *Class II Positive Control Serum* (mixtures of sera with known antibodies against HLA class I or II)[46]. These often come with a certificate of reactivity. Another high-quality option is to use NIBSC standards, e.g. *NIBSC Strong Positive Plasma, Ref. 17/212*, which contains antibodies against multiple HLA (suitable for use as a control in FCXM and SAB-Luminex)[42]. In addition, some institutions maintain their own internal pool of positive sera (periodically updated with samples from patients with high PRA) as a routine control.

In all cases, it is critical to keep a **batch record** of the controls used, their expected results, and the results obtained. If a control fails (e.g., the positive does not mark when it should, or the negative exhibits abnormal fluorescence), it must **be investigated before continuing**, which may involve repeating the assay with alternative controls. Using good quality controls and following these recommendations ensures that the XMStep kit produces reliable and clinically interpretable results. As the guidelines state: *"A crossmatch should always include appropriate controls; a well-characterised negative control serum to set the baseline in FL1, and a positive control serum to serve as a positive marking reference"*[17][18]. In this way, the laboratory and clinician can be confident that a **"negative crossmatch"** truly means the absence of relevant antibodies, and that a **"positive crossmatch"** is a reproducible finding that warrants attention in patient management.

References: (Included throughout the text with identifiers 【】, highlighting Immunostep technical documentation and key literature).

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