VALIDATION/VERIFICATION & CHANGE CONTROL PROFORMA

Supplemental data

This form is intended as a guide to aid laboratories in developing a suitable validation/verification procedure. A suggested methodology for using the form has been given below.

The terms 'investigating scientist' and 'senior scientist' have been used for illustration of an authorisation procedure – organisation of authorisation systems is a matter for individual laboratories.

1. Section 1 should be completed as far as possible to establish the goals and general format of the validation/verification/change control.
   - Sections 1.1 "**Intended use or application**" and 1.2 "**Requirements**" *must* be completed at the start of the procedure. The assessment of the validation/verification depends formally on confirmation, through the provision of objective evidence, that these requirements have been fulfilled[i].
   - If mentioned (1.2), the "Expected Performance" should be distinguished from the "Requirements", which must be shown to have been fulfilled.
     E.g. *The statement "should detect all known point mutations of haemophilia A" could be included as a guide in the Expected performance; if stated as a requirement, however, it would need to be proved.*
2. Section 2 covers the validation of utility which should be carried out for all validations and verifications. In the majority of cases this section can be completed on objective evidence from developmental work, design procedures (e.g. SNP checking primers) or by the use of limitations or controls in the on-going test. Where this is not the case, work plans for relevant parameters should be prepared as in 3 below.
3. Appropriate parameters for experimental investigation should be identified with the aid of the table appendix A – a checklist is also provided at the top of section 3. For each parameter required, the investigating scientist develops a work plan based on section 3 (these are referenced 3.1, 3.2 to 3.*n*) by completing copies of sections 3.*n*.1 ('Aims', 'Samples' and 'Methodology'). It is suggested that these be maintained in a single document.
   Note*: several parameters may be tested in a single experiment, for example sensitivity and specificity.*
4. The work plan[s] should be agreed and authorised by the investigating and the senior scientist by signing and dating in the boxes provided.
5. The experimental work is performed and analysed by the investigating scientist who should then complete the 'experimental results' and 'interpretation' sections 3.*n*.2.
6. The 'outcome and limitations' should be agreed between the investigating and senior scientists by signing and dating in the boxes provided.
7. Points 3 to 6 should be repeated for each parameter to be tested.
8. If there is any non-compliance between the experimental results and the required performance specification detailed in section 1.2 the parameter in question should be re-examined to determine if the methodology can be changed or new limitations introduced to rectify the non-compliance. Any further work should be recorded in a new section 3 work plan. Alternatively the implementation can be abandoned.
9. Once all the parameters have been satisfactorily investigated the investigating and senior scientist can agree and sign off the final conclusions in section 4.
10. Assuming the validation/verification has been completed satisfactorily an implementation plan can be drawn up. Appendix B provides a basis for an administrative checklist for the implementation.

[i] (ISO 9000:2005 3.8.4, 3.8.5)

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1. Validation/verification[i] details

Test name	Validation of Mismatch Data Aggregator software	**Q-Pulse Reference**	TT.VAL077

1.1 Test details

Intended use or application	The 'Mismatch data aggregator' compares donor and recipient HLA types to identify serological and molecular donor mismatches, as well as identifying the following: • Highest MFI (and date) of DSA (donor specific antibody) for each mismatch. • All dates and corresponding MFI values for each mismatch. The software was developed with the help of Afzal Chaudhry and is intended for research use to study DSA development in transplant patients.
Locus / Gene / Marker	Donor and recipient HLA types, and recipient Luminex SAB data (dates and MFI levels for each specificity) processed by 'Patient_SAB_combiner_0_9_december_2020' formally validated as 'anonymiser_0_9_december_2020' (TT.VAL071)
Reference Sequence	N/A
Outline methodology	10 randomly selected patients from two different cohorts of transplant patients will be identified - 5 from cardiac, and 5 from intestinal. Independent analysis of 5 patients within each cohort will take place. Two validation folders, one for cardiac validation and one for intestinal, will each contain a 'Mismatch data aggregator' comprising recipient and donor HLA types and combiner files of recipient Luminex SAB data for that cohort of patients. Aggregator will generate output sheets Data_1, Data_2 and Data_3 which will be checked against manually identified mismatches recorded in a table. By making comparisons to combiner files, checks will be performed to ensure the aggregator correctly identifies DSA with corresponding dates and MFI levels. One completely HLA antibody negative patient will be tested in each cohort to ensure empty combiner files are still processed correctly.
SOP	N/A – this is a research tool.
References	Random number generator: https://www.random.org 'Patient_SAB_combiner_0_9_december_2020' and associated validation document 'TT.VAL071 Validation of Normalised Combiner (anonymiser software for combining Luminex patient data)'

[i] Delete as appropriate

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1.2 Validation details

Overall Aims	To ensure completeness and accuracy of sample data generated by 'Mismatch Data Aggregator' by comparison with donor and recipient HLA types and Luminex SAB data combined using 'Patient_SAB_combiner_0_9_december_2020'. If data matches, the Mismatch Data Aggregator can be used for research with confidence of functional accuracy.
Requirements	1. Donor mismatches should be been correctly identified at: • A, B, C, DRB1, DRB3/4/5 and paired DQA1/DQB1 (Molecular). • A, B, Cw, DR, DRB3/4/5 and DQ (Serological). 2. Dates and MFI data for each mismatch are identical to those in the patient combiner file (made using 'Patient_SAB_combiner_0_9_december_2020'). The output results sheets must contain: • Data 1: Highest MFI and date for each mismatch (serological and molecular). • Data 2: All dates and MFI values for a molecular mismatch. • Data 3: All dates and MFI values for a serological mismatch. 3. Missing HLA typing data, matched HLA types, no SAB data, negative DSA tested data, and homozygous donor HLA types should be handled appropriately and be identifiable from output results sheets.
Validation/verification	Validation (new in-house software).
Type	Validation performed prior to implementation.
Scope / limitations	None
Turnaround time	N/A
Other considerations	Accuracy of 'Patient_SAB_combiner_0_9_december_2020' – assessed in TT.VAL071. Accuracy of high resolution HLA type assimilator (used to predict high resolution HLA types). Updates to Excel may affect software function.

2. Validation of Utility

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Test name	Validation of Mismatch Data Aggregator software	**Q-Pulse Reference**	TT.VAL*XXX*

Applicability of measurements	Accuracy of output data from 'Mismatch Data Aggregator' is appropriate and sufficient to achieve the desired results.
Selectivity	There are no selectivity issues, limitations or control measures required to ensure test utility.
Interferences	None
Cross-reactivity	N/A

Authorisation	Name	Signature	Date
Investigating scientist	Rebecca Cope & Rhea Langeveld		29/11/2021
Senior Scientist (Authorisation)	Sarah Peacock		20/12/21

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3. Validation of Mismatch Data Aggregator

A copy of this section should be filled in for all parameters to be tested.

Test name	Validation of Mismatch Data Aggregator software	**Q-Pulse Reference**	TT.VAL*XXX*

☐ Sensitivity	☐ Trueness	☐ Reproducibility	☐ Limit of quantification
☐ Specificity	☐ Repeatability	☐ Robustness	☐ Linearity
☒ Accuracy	☐ Intermediate precision	☐ Limit of detection	☐ Measurement uncertainty

3.1 Work plan

Section aims	To ensure that serological and molecular HLA mismatches are correctly identified by the 'Mismatch Data Aggregator' and the generation of data in the output results sheet is accurate: • Data 1: Highest MFI and date for each mismatch (serological and molecular). • Data 2: All dates and MFI values for a molecular mismatch. • Data 3: All dates and MFI values for a serological mismatch. Manual comparisons will be made on the recipient and donor HLA types to identify mismatches, and comparisons will be made to combiner files on each patient vs. the 'Mismatch Data Aggregator' output results sheets. To check that missing HLA typing data, matched HLA types, no SAB data, negative DSA tested data, and homozygous donor and recipient HLA types are handled appropriately by the 'Mismatch Data Aggregator' and be identifiable from the output results sheets. Essential data that is required by the 'Mismatch Data Aggregator' to function correctly will be identified.
Samples	Random number generator will be used to select 10 patients, 5 from Intestinal and 5 from Cardiac research cohorts. One from each of the 5 selected patients should be a completely negative patient with no SAB data. Record of all patients, samples tested, combiners and validation notes/workflow is available in Z:\Tissue Typing\Quality Management\Change Control & Validation Forms (SOP 133)\Validation of Mismatch Data Aggregator.
Methodology	1. Make copy of selected patient combiners in cohort validation folder. 2. Copy and paste 'Mismatch Data Aggregator' into folder. 3. Copy HLA types of all patients in cohort from converter and paste (values) into Data sheet of 'Mismatch Data Aggregator'. Converter sheet where copying HLA types from must be set up in same format as Data sheet, with correct column headers and hidden columns the same. Delete rows of data of patients not required in validation leaving only those 5 selected patients HLA types. 4. In CALC sheet, enter '5' in cell B3. 5. Run aggregator. 6. Manually work out and list serological and molecular mismatches in 'Serol MM' and 'Mol MM' columns in each patient table in 'Validation of aggregator notes' document. • Insert row underneath patient HLA type in Data sheet, copy entire donor HLA type directly below. • Highlight mismatched donor HLA.

	• Add mismatches to table. 7. Checks are performed for each locus as follows: • Data_1: Check highest serological and molecular DSA has been correctly identified from combiner – check donor MM, MFI and date. • Data_2: Check all DSA & dates for molecular data have been correctly identified from combiner. • Data_3: Check all DSA & dates for serological data have been correctly identified from combiner. 8. Mark data columns in table with 'Y' when checks complete and correct for each locus. • For discrepancies, add details. • Add comments underneath tables.

Authorisation	Name	Signature	Date
Investigating scientist	Rebecca Cope & Rhea Langeveld		29/11/2021
Senior Scientist (Authorisation)	Sarah Peacock		20/12/2021

3.2 Partial results and conclusions

Experimental results

During initial phases of testing for patient 1826951 who has lots of SAB I and II data (approximately 60 samples), including mismatch antibody data where there were 'x's present (which indicate where no MFI data is present as the sample was tested on a SAB lot without that molecular specificity of that antigen), the following issues were spotted:

- Data 1 - highest C*04:01 and Cw4 not pulled through
- Data 2 - not all C*04:01 samples and corresponding MFI points pulled through
- Data 3 - same Cw4 samples not pulled through as above, plus another Cw4 that is molecularly different (C*04:03), but not serologically different is not pulled through

Sent problems to Afzal on 15/11/2021 and these were quickly resolved. The issue related to presence of 'x's disrupting the coding, causing a premature exit from the algorithm. Afzal returned a new version of the aggregator on 16/11/2021 which fixed all these issues. All patient data in this validation (1-10) was reran using the updated 'Mismatch Data Aggregator'.

Using the updated 'Mismatch Data Aggregator', all data produced by the aggregator was present and accurate – see 'Validation of aggregator notes' document saved Z:\Tissue Typing\Quality Management\Change Control & Validation Forms (SOP 133)\Validation of Mismatch Data Aggregator\TT.VALXX MDA.

During the validation process we clarified the rules by which the 'Mismatch Data Aggregator' processes data:

1. If there is a mismatch and DSA present, this will be displayed as, for example (peak MFI in Data 1, multiple sample dates and corresponding MFIs in Data 2 and 3):

MM_HLA_Typing	MM_HLA_DATE	MM_HLA_MFI
A2	29/11/2021	231

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2. Where there is no mismatch this will be denoted as:

MM_HLA_Typing	MM_HLA_DATE	MM_HLA_MFI
xxx	xxx	xxx

3. Where a patient is completely negative for HLA antibodies (no SAB testing performed) or no MFI data (no bead) is present but a mismatch is present, the mismatch will be stated but the other fields will contain, for example:

MM_HLA_Typing	MM_HLA_DATE	MM_HLA_MFI
A2	xxx	xxx

4. Where a patient is negative for DSA at a particular mismatch but has other HLA antibodies, this will be denoted as (for each date of SAB testing including that specificity):

MM_HLA_Typing	MM_HLA_DATE	MM_HLA_MFI
A2	01/01/2001	0

5. Where HLA typing data is missing, this will be denoted 'ND' (ND=no data) as a means to flag missing data as a possible mismatch:

MM_HLA_Typing	MM_HLA_DATE	MM_HLA_MFI
ND	xxx	xxx

For missing recipient data, both donor mismatches will be flagged ND, even if only one allele/antigen is missing.
For missing donor data, ND will be present for whichever allele/antigen is missing.

Except for DRB3/4/5, where missing and matched data will be denoted by 'xxx'. The aggregator functions this way as not all DRB1s have an associated DRB3/4/5 (e.g. DR1) so 'ND' is not appropriate here.

MM_HLA_Typing	MM_HLA_DATE	MM_HLA_MFI
xxx	xxx	xxx

Users should ensure DRB3/4/5 HLA data is fully complete as the aggregator cannot distinguish between missing data (in error/incomplete) or correctly absent DRB3/4/5 because of the DRB1 associated. If this is ensured then it can be assumed that 'xxx' is either flagging correctly absent DRB3/4/5 or a match – neither of which require any concern with regards to DSA.

6. For homozygous donors:

- If there is SAB data present, a single mismatch will be displayed in Data 2 & 3. E.g. The A locus will display only MM_A1.
- If there is no SAB data (no bead, or the recipient is non-sensitised) or the donor is matched, the mismatch will be displayed twice in Data 2 & 3. E.g. The A locus will display MM_A1 and MM_A2.

7. In Data 1, if there are multiple samples with the same highest DSA MFI level, the date of the sample pulled through in Data 1 is the date of the sample that appears first in the samples listed in the combiner (which isn't necessarily the most recent date). This might

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	typically occur if there is a MM but no DSA in the patient history (and they have antibodies at other specificities), and MFI at that MM will be 0 on multiple occasions. Ideally the most recent MFI date with that highest MFI would be pulled through in order to have the most contemporary date of that DSA. To circumvent this issue, the combiner file can be formatted so that samples are listed by date (they are currently listed by Session ID). We identified that it would be good practice to check combiner files to ensure that the following applies before the 'Mismatch Data Aggregator' is used, as the data in the combiner is essential to accurate functioning and ease of use of the aggregator: • Ensure all SAB samples in combiner have a date (date can sometimes be inferred from sample ID) • Order SAB samples in combiner by date • Remove repeat and diluted repeat samples from combiners to avoid duplicated repeated dates in aggregator. • Format MFIs to nearest whole number for ease of viewing if this is the user's preference We identified that the following essential data is required by the 'Mismatch Data Aggregator' to function correctly: • J = TX_ID: Patient ID • K = TX_DATE: 1/1/2000 • AN = tx_date2: Today's date • Split columns: Serological HLA type* • HR_Split columns: High resolution molecular HLA type* * Note: if donor data is missing/incomplete, the 'Mismatch Data Aggregator' will still function and process the data that is available – e.g. if one allele is missing, the other will be processed to check if a mismatch. Recipient data however, requires a full serological or molecular HLA type to check for mismatches (ideally both for full output). Note: Lack of DRB3/4/5 due to associated DRB1 (e.g. DR1) will not stop the aggregator functioning.
Interpretation	The 'Mismatch Data Aggregator' reached its objective in accurately identifying serological and molecular HLA mismatches at A, B, Cw, DR, DRB3/4/5 and DQ by comparing donor to recipient HLA typing data. • Data 1 is able to identify the highest MFI of serological and molecular DSA. • Data 2 is able to pull through all dates and MFI values for a molecular mismatch. • Data 3 is able to pull through all dates and MFI values for a serological mismatch. Missing HLA typing data, matched HLA types, no SAB data, negative DSA tested data, and homozygous donor HLA types are handled appropriately and identifiable from output results sheets – with the exception of DRB3/4/5, where both missing (because of error/incomplete or correctly absent DRB3/4/5 because of the DRB1 associated) and matched data is denoted by 'xxx'.
Outcome / limitations	The results fulfil the validation requirements. A set of instructions for use of the 'Mismatch Data Aggregator' has been produced and instructions have been inputted into the 'INPUT', 'CALC' and 'RESULTS' sheets of the 'Mismatch Data Aggregator'.

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	Limitations: • Do not process >200 patients at any one time (more will cause Excel to crash). • Single Antigen Beads, and their corresponding HLA antibody specificities, are not consistent across all samples tested over a wide period of time. When the lot number changes, this may result in different beads being included in the SAB tests. Therefore a patient may have had HLA antibodies to particular specificity on one lot but then this specificity is no longer tested on a future lot so the MFIs will no longer be listed. • Input of high resolution HLA typing data from an inaccurate HLA type assimilator (used to predict high resolution HLA types) – this will limit the accuracy of the mismatch data produced and DSAs identified if the high resolution HLA types of donor and recipient are inaccurate. • The Mismatch Data Aggregator is intended to be used in combination with 'Patient_SAB_combiner_0_9_december_2020', which combines Luminex single antigen bead (SAB) data output files from One Lambda's HLA Fusion software into a single document. We have not validated the Mismatch Data Aggregator working with combined SAB data from any other manufacturer. Further tweaks that could improve the 'Mismatch Data Aggregator' in the future: • The aggregator could be coded so that the most recent DSA MFI and date could be pulled through in Data 1 to avoid formatting this in each combiner. • The aggregator could be coded so that MFIs are rounded to the nearest whole number to avoid formatting this in each combiner - little is to be gained from the current 2 decimal place MFI values given the imprecision of the Luminex assay itself. • There is no ':' separator between DQ first and second fields. E.g. DQA1*05:01,DQB1*02:01 is written DQA1*0501,DQB1*0201. • Distinction in the DRB3/4/5 fields for missing data (in error/incomplete) or correctly absent DRB3/4/5 because of the DRB1 associated.

Authorisation	Name	Signature	Date
Investigating scientist	Rebecca Cope & Rhea Langeveld		29/11/2021
Senior Scientist (Authorisation)	Sarah Peacock		20/12/21

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4. Validation Final Conclusions

Test name	Validation of Mismatch Data Aggregator software	**Q-Pulse Reference**	TT.VAL*XXX*

Overall Conclusion	The 'Mismatch Data Aggregator' is able to accurately identify serological and molecular HLA mismatches at A, B, Cw, DR, DRB3/4/5 and DQ by comparing donor to recipient HLA typing data. Output in: • Data 1 is able to identify the highest MFI of serological and molecular DSA. • Data 2 is able to pull through all dates and MFI values for a molecular mismatch. • Data 3 is able to pull through all dates and MFI values for a serological mismatch. The output data on each patient is standardised and can be used for analysis on a large cohort of patients (up to 200). The 'Mismatch Data Aggregator' safe to use in research.
Estimates of accuracy and measures of uncertainty	**N/A** **Give experimentally-derived values for the relevant metrics. Comment on the potential influence of the uncertainty on the reliability of the result.**
Limitations and/or predictable interferences	**Listed above in outcome/limitations section**
Internal QC	**N/A**
External QA	**N/A**

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Investigating scientist	Rebecca Cope & Rhea Langeveld		29/11/2021
Senior Scientist (Authorisation)	Sarah Peacock		20/12/21

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Reference: Mattocks CJ, Morris MA, Matthijs G, et al. A standardized framework for the validation and verification of clinical molecular genetic tests. European Journal of Human Genetics. 2010;18(12):1276-1288. doi:10.1038/ejhg.2010.101 (supplementary material) available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3002854/

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Appendix A: Types of test. (Refer to main paper for full descriptions of test types)
*NB. In addition to the parameters detailed below appropriate **robustness** testing should be carried out for all types of test.*

	Description	Examples	Sensitivity[a]	Specificity[b]	Accuracy[c]	Trueness	Precision[d]	Limits of detection	Probability[e]
A	**Quantitative** tests. The result can have any value between two limits (including decimals).	Determination of methylation load (%); characterization of a mosaic mutation; heteroplasmy of mitochondrial variants.				++	++	++	
B	**Categorical** tests where the quantitative signal is placed into an ordinal series to give the final result.	Sizing a PCR product; determination of triplet repeat size (FRAXA, Huntington disease, etc.)			+	++	++	++	+
C	**Categorical** tests where the quantitative signal is placed into one of a limited series of predefined categories to give the final result.	Determination of copy number using PCR or MLPA.: exon deletion / duplication in *BRCA1*; *PMP22* gene dosage in CMT and HNPP;			+	To establish correction factors and/or cut-offs			++
D	**Qualitative** tests where the true quantitative signal can have one of many possible values, but the required result can only have one of two possible values.	Mutation scanning for unknown mutations e.g. by sequencing or high resolution melt.	++	++	+	To establish correction factors and/or cut-offs		++[f]	
E	**Qualitative [binary]** tests where the true quantitative signal can only have one of two possible values	Genotyping for a specific mutation e.g. *CFTR* Phe508del in cystic fibrosis or *HFE* Cys282Tyr in hemochromatosis.	++	++	+	To establish correction factors and/or cut-offs		++[f]	+

Legend

	Metric used for implementation validation
	Metric used for implementation or ongoing validation
	Metric used for ongoing validation
++	Recommended parameter
+	Applicable parameter (less used)

Notes

a.	Sensitivity = True Positive / (True Positive + False Negative)
b.	Specificity = True Negative / (True Negative + False Positive)
c.	Accuracy = True Result / (True Result + False Result)
d.	Precision should be measured in terms of repeatability and intermediate precision (as well as reproducibility for inter-laboratory validations)
e.	The term 'probability' is used to describe situations where a probability that the result is correct can be assigned – primarily in ongoing validation (e.g. competitive hypothesis testing)
f.	Should be used in tests where genotyping of low level variations is required for example mitochondrial DNA

Appendix B: Administrative checklist

- ☐ Validation completed and approved
- ☐ Complete SOP
- ☐ Order reagents
- ☐ Health and safety aspects (personal, reagents)
- ☐ Equipment (electrical testing, maintenance)
- ☐ Subscribe to EQA
- ☐ Update request forms
- ☐ Update website and any directory listings
- ☐ Billing procedure
- ☐ Training
- ☐ LIMS functionality
- ☐ Worksheets
- ☐ Inform clients
- ☐ Report template

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