Recommendations for the determination of measurement uncertainty for assays performed in the histocompatibility and immunogenetics (H&I) laboratory

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1. Definition of measurement

A measurement generally tells us the property of something, such as how heavy, how hot or how long an object is. It is usually in two parts: a number and a unit of measurement.

It is also useful to define what is not a measurement, for instance comparing two items, counting or a yes/no or pass/fail test result (although measurements may be part of the process leading up to the result).

2. Definition of measurement uncertainty (MU)

The MU is the doubt that exists about the result of any measurement. Uncertainty can be systematic or random and arise in the pre-analytical, examination or post-analytical phases of a process. The following may contribute to the level of uncertainty associated with a measurement:

- the measuring instrument
- the item being measured
- the measurement process
- imported uncertainties (e.g. calibration of instrument)
- operator skill (not gross mistakes)
- sampling issues (during the measurement process)
- the environment.

These are individual inputs that contribute to the overall uncertainty in the measurement.

Measurement of uncertainty is not:

- operator mistake
- tolerances
- specifications
- accuracy
- errors
- statistical analysis.

3. Calculation of MU

To calculate MU, the sources of uncertainty in the measurement must first be identified.

Second, the size of the uncertainty from each source should be estimated.

These are then combined to give the individual uncertainties an overall figure. This can be derived in two ways:

- type A – uncertainty estimates using statistics
- type B – uncertainty estimates from any other information (such as past experience, manufacturer specifications, published information and common sense).

Eleven steps to evaluating uncertainty

1. Decide what you need to find out from your measurements.

2. Decide what actual measurements and calculations are needed to produce the final result.

3. Carry out the measurements needed.

4. Estimate the uncertainty of each input quantity.

5. Express all uncertainties in similar terms.

6. Decide whether the errors of the input quantities are independent of each other.

7. If they are not independent, then extra calculations are needed.

8. Calculate the result of your measurement (include known corrections for things such as calibration).

9. Find the combined standard of uncertainty from all the individual elements.

10. Express the uncertainty in terms of a coverage factor together with a size of the uncertainty interval and state a level of confidence.

11. Write down the measurement result and the uncertainty and state how you got these.

4. Assays relevant to histocompatibility and immunogenetics (H&I)

Below is a summary of assays relevant to H&I and recommendations as to how to approach MU in the laboratory.

a. PCR-SSP

Given the nature of the results generated by this assay, statistical determination of the MU is not required. However the laboratory should demonstrate that they have considered the potential sources of uncertainty that could contribute to the assay (see Table 1 below for an example).

b. Luminex-based HLA typing

Given the nature of the results generated by this assay, statistical determination of the MU is not generally required. However the laboratory should demonstrate that they have considered the potential sources of uncertainty that could contribute to the assay (see Table 2 for blank outline).

c. Sequence-based HLA typing

Given the nature of the results generated by this assay, statistical determination of the MU is not required. However the laboratory should demonstrate that they have considered the potential sources of uncertainty that could contribute to the assay (see Table 2 for blank outline).

d. Complement dependent cytotoxic assays

Given the nature of the results generated by this assay, statistical determination of the MU is not required. However the laboratory should demonstrate that they have

considered the potential sources of uncertainty that could contribute to the assay (see Table 2 for blank outline).

e. Flow cytometry

The laboratory should demonstrate that they have considered the potential sources of uncertainty that contribute to the assay (see Table 2 for blank outline and Appendix A for a worked example). The data generated by this assay is numerical and semi-quantitative and whilst results that are reported to clinicians will have been interpreted in reference to local policy, statistical determination of MU is nevertheless required. This requirement is greater where any assessment of level of reactivity is made.

f. Luminex-based antibody screening results

The laboratory should demonstrate that they have considered the potential sources of uncertainty that could contribute to the assay (see Table 2 for blank outline). The data generated by this assay is numerical and semi-quantitative and a statistical determination of the MU is required. This should, as a minimum, provide longitudinal evidence of assay accuracy and bias.

g. Chimerism analysis

The laboratory should demonstrate that they have considered the potential sources of uncertainty that could contribute to the assay resulting in a quantitative MU (see Appendix B for a worked example). Given the quantitative nature of the results generated by this assay, statistical determination of the MU is required and should, as a minimum, provide an assessment of the potential maximum level of error associated with a reported result.

h. Next-generation sequencing

Given that this is a newly emerging technology for the field of H&I, the recommendations for consideration of MU are to consider in the same way as for sequence-based typing.

Table 1: Example of consideration of MU for PCR-SSP

Source of uncertainty	Description of uncertainty	Effect of uncertainty	Risk of uncertainty			Mitigation of uncertainty
			Likelihood	Severity	Final score	
Measuring instrument	HLA typing kits/ 'in-house' batches	Data confidence				Batch testing of commercial kits. Verification of 'in-house' primer batches.
Item being measured	The result generated is a yes/no result	Data confidence				Competency evaluation.
Measurement process	The result generated is a yes/no result	Data confidence				Competency evaluation.
Imported uncertainties	Addition of PCR mastermix	Assay sensitivity				Use of calibrated pipettes.
	Addition of DNA	Assay sensitivity				Use of calibrated pipettes.
	Dispensing of DNA/ mastermix into PCR plate	Assay sensitivity				Use of calibrated pipettes. Use of electronic pipettes.
	Running PCR programme	Assay sensitivity/ specificity				Annual maintenance and regular monitoring of thermal cyclers.
	Agarose gel electrophoresis	Data confidence				Working to SOP. Use of DNA ladder.
Operator skill (not gross mistakes)	Interpretation	Data confidence				Working to SOP. Criteria for interpretation validity. Use of internal controls. Participation in continuing competency and EQA.
Environment	Avoiding contamination	Data confidence				Separation of pre- and post-PCR. Use of water control. Regular contamination testing.
Sampling issues	Low-concentration or poor-quality DNA will affect the performance of the test. The laboratory has established mechanisms in the SOP for handling results from these samples	Data confidence				Ensuring proper collection of samples by having sufficient information for users. Use of Nanodrop spectrophotometer to determine DNA concentration.

Risk assessment that could be used

Likelihood

Value	Term	Other description
1	Very low (0–5%)	Unlikely to occur
2	Low (6–20%)	Incident likely to occur once in a five-year period
3	Medium (21–50%)	Incident likely to occur yearly
4	High (51–80%)	Incident likely to occur once in a six month period
5	Very high (81–100%)	Incident likely to occur every month or more frequently

Severity

Value	Definition
1	No patient impact
2	Delayed result
3	No result and/or request for repeat sample
4	Incorrect result issued, no treatment impact
5	Incorrect result issued, incorrect treatment impacting on patient health

Table 2: Blank outline which could be used for all assays

Source of uncertainty	Description of uncertainty	Effect of uncertainty	Risk of uncertainty			Mitigation of uncertainty
			Likelihood	Severity	Final score	
Measuring instrument						
Item being measured						
Measurement process						
Imported uncertainties						
Operator skill (not gross mistakes)						
Environment						
Sampling issues						

Appendix A: Worked example

Central Manchester University Hospitals NHS
NHS Foundation Trust

TRANSPLANTATION LABORATORY
MANCHESTER ROYAL INFIRMARY

Determination of the measurement of uncertainty for the flow cytometry crossmatch

MoU Report 02/07/2015

Report completed by Amanda Robson

Report Number	Checked by	Date

Contents

Appendix A: Worked example

Summary

The standard uncertainty for the measurement of the negative control value is a linear channel shift of 1.29 for T cells and 1.84 for B cells

This measurement is derived from the document ”M3003 The Expression of Uncertainty and Confidence in Measurement Edition 3 November 2012 UKAS”.

Action plan			
Key action(s)	Co-ordinator for action	Timescale	Completion date
To be re-evaluated once per year once the FC500 has had the annual PM visit.	PF	October 2015	
To be re-evaluated each time a negative control batch changes.	PF	As necessary	

Introduction

The laboratory is required to determine the measurement of uncertainty for tests which are used to report a measured quantity value on a patient sample. The performance requirements for each measurement procedure will be defined and regularly reviewed. All the factors which could influence the measurement and subsequent report result have to be accounted for, and estimates made of the potential variation associated with these factors. This will allow the laboratory to rely on statistical data from repeated measurements in order to state the degree of confidence that a measured value lies within a repeated range.

Several measurements will have to be made in order to obtain a realistic reading by using the arithmetic mean. Only a finite number of measurements can be made, which introduces an uncertainty which is represented by the difference from our calculated mean value and the underlying “true” mean value. A statistical approach will therefore be undertaken to determine how far the calculated mean is away from the “true” mean. The calculated uncertainty will be referred to as “**the experimental standard deviation of the mean or U**”, and repeated as necessary.

Aim

To calculate the uncertainty for the flow cytometry crossmatch described in SOP XM022 (Analysis of Crossmatch Results from the FC500 Flow Cytometer).

The relevant uncertainty components will be listed in Table 1 below commencing from the sample arriving in the laboratory, testing and measurement procedure, and reporting the measured value.

Appendix A: Worked example

Table 1: Sources of uncertainty and their effect

Source of uncertainty	Estimate of effect	Addressed by
Input cell count	Assay sensitivity	Use of counting chamber to determine cell number
Input cell volume	Assay sensitivity	Use of calibrated pipettes
Input serum volume	Assay sensitivity	Use of calibrated pipettes
Incubation times	Assay sensitivity/specificity	Working to SOP
Cell washing	Assay sensitivity/specificity	Use of pre-set programme on centrifuge
Flow cytometer performance	Assay sensitivity	Daily calibration
Event count	Data confidence	Use of standard protocol
Transcription of results and calculations	Variable	Rigorous checking procedure employed to check all numbers and automatic generation of relevant calculations

Method

Flow cytometry crossmatch analysis is performed by comparison of a sample against a negative control. The median fluorescence of the negative control sample crossmatched against donor lymphocytes is the particular quantity being measured and will be known as the “measurand”. The measurement of uncertainty will be considered and how it might impact on clinical samples. A series of calculations will be undertaken which are “Type A” evaluation of uncertainty. Type A evaluations are used to estimate random errors arising from random variations of the test. A number of sources may contribute to the variability each time a measurement is taken which cannot be eliminated. The variability can be estimated by using statistical analysis of a series of observations which are used to obtain a value for the repeatability of a process.

The reproducibility of the procedure will be determined by using a single control serum crossmatched against a single donor cell preparation, repeating the measurement 20 times within the same crossmatch. This will estimate the variation in the analysis which could be due to minor variations in patient antibody binding to donor lymphocytes. A lymphocyte preparation originating from a blood sample is homogeneous and measurements on twenty aliquots from one single blood sample crossmatched against the same control serum should allow the Laboratory to assess whether a difference in results contributes to the uncertainty of the measurement procedure.

The estimated standard deviation is calculated using the values which have been measured (median fluorescent channel for T cell and for B cells). The experimental standard deviation of the mean is then obtained by dividing this value by the square root of the number of measurements that contributed to the mean value.

Appendix A: Worked example

Results

The results from Table 2 indicate the results from the reproducibility of the procedure. This has encompassed factors which affect uncertainty. The results are shown as sample number, mean fluorescent channel, calculated linear channel shift and estimated standard deviation. The standard uncertainty has been determined by using the following calculations:

Conversion of median fluorescent channel to linear channel

$$= \text{Log}^{10}(10 \times \text{median}) \times 256$$

$$U = \frac{\text{standard deviation}}{\sqrt{n}}$$

Appendix A: Worked example

Table 2: results from the reproducibility of the flow cytometry crossmatch using the mean fluorescent channel reading from 20 identical crossmatch samples

Sample number	T cell median fluorescent channel	T cell linear channel	B cell median fluorescent channel	B cell linear channel
1	0.671	211.64	2.36	351.47
2	0.641	206.56	2.16	341.62
3	0.683	213.61	2.24	345.66
4	0.653	208.62	2.3	348.60
5	0.613	201.59	2.08	337.42
6	0.647	207.59	2.12	339.54
7	0.63	204.63	2.12	339.54
8	0.624	203.57	2.08	337.42
9	0.636	205.69	2.03	334.72
10	0.619	202.67	1.92	328.53
11	0.624	203.57	2.03	334.72
12	0.641	206.56	2.03	334.72
13	0.613	201.59	1.99	332.51
14	0.571	193.70	1.87	325.59
15	0.641	206.56	1.97	331.38
16	0.576	194.67	1.84	323.79
17	0.571	193.70	1.94	329.68
18	0.619	202.67	1.74	317.58
19	0.653	208.62	2.08	337.42
20	0.683	213.61	1.99	332.51
Mean	0.630	204.71	2.04	335.51
Standard deviation		5.76		8.22
Standard uncertainty = U		1.29		1.84

The standard uncertainty for the measurement of the negative control value is a channel shift of 1.29 for T cells and 1.84 for B cells.

This is recorded on the analysis protocol for flow cytometry crossmatching.

Appendix B: Worked example

Central Manchester University Hospitals NHS
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TRANSPLANTATION LABORATORY
MANCHESTER ROYAL INFIRMARY

Determination of the measurement of uncertainty for the chimerism monitoring technique

MoU Report 17/06/2015

Report completed by Helena Lee

Report Number	Checked by	Date
2		

Contents

Appendix B: Worked example

MoU Outcome: Summary		Report number	2
Project title	**Determination of the measurement of uncertainty for the chimerism monitoring technique**		

The value of the measurand in % donor for the NEQAS IQA 152 DNA sample is 84.5% donor +-0.3% donor.

"The reported expanded uncertainty is based on standard uncertainty multiplied by a coverage factor k=2, providing a coverage probability of approximately 95%. The uncertainty evaluation has been carried out in accordance with UKAS requirements. This measurement is derived from the document M3003, *The Expression of Uncertainty and Confidence in Measurement (Edition 3*), November 2012 UKAS".

Action plan			
Key action(s)	Coordinator for action	Timescale	Completion date
To be re-evaluated once per year once the ABI 3500XL has had the annual service.	HEL	Service due January 2016	
To be re-evaluated each time a reagent batch changes.	HEL	As necessary	

Introduction

The laboratory is required to determine the measurement of uncertainty for tests which are used to report a measured quantity value on a patient sample. The performance requirements for each measurement procedure will be defined and regularly reviewed. All the factors which could influence the measurement and subsequent report result have to be accounted for, and estimates made of the potential variation associated with these factors. This will allow the laboratory to rely on statistical data from repeated measurements in order to state the degree of confidence that a measured value lies within a repeated range.

Several measurements will have to be made in order to obtain a realistic reading by using the arithmetic mean. Only a finite number of measurements can be made, which introduces an uncertainty which is represented by the difference from our calculated mean value and the underlying "true" mean value. A statistical approach will therefore be undertaken to determine how far the calculated mean is away from the "true" mean. The calculated uncertainty will be referred to as "**the experimental standard deviation of the mean or u**", and repeated as necessary. The independent components are then squared and added together, and the square root is then taken. The resulting combined standard uncertainty is multiplied by a coverage factor k to provide the expanded Uncertainty, **U**.

Appendix B: Worked example

Aim

To calculate the expanded uncertainty for the chimerism monitoring procedure as described below in the Method section (SOP IB001, IB020, IB007, IB008). The relevant uncertainty components will be listed in Table 1 below commencing from the sample arriving in the laboratory, testing and measurement procedure, and reporting the measured value.

Table 1: Sources of uncertainty and their effect.

Source of uncertainty	Estimate of effect	Addressed by
The environment: temperature of instrument, current applied to ABI 3500XL	Unknown – may affect peak heights/area of post-PCR product	Weekly calibration of thermal cyclers, yearly service of ABI 3500XL
Sampling issue: calibration of pipettes	Unknown – may affect peak heights/area of post-PCR product	Use of calibrated pipettes
Operator's training and competence	Unknown – may affect peak heights/area of post-PCR product	Trained and signed off on relevant SOPs
Reagent batch change	Unknown – to be evaluated as necessary	Intra-assay and inter-assay variation estimated (see Method)
Changes in the characteristics or performance of the ABI 3500Xl since the last calibration/service	Unknown – to be evaluated once per year	Intra-assay and inter-assay variation estimated (see Method)
Intra-assay variation	Unknown – may affect peak heights/area of post	20 measurements made of % donor within one PCR originating from a single DNA sample
Inter-assay variation	Unknown – may affect peak heights/area of post	% donor measured using a single DNA sample from 20 different assays
Transcription of numbers and calculations	Variable	Rigorous checking procedure employed to check all numbers and relevant calculations

Method

The percentage donor of a single DNA sample is the particular quantity being measured and will be known as the 'measurand'. The measurement of uncertainty will be considered and how it might impact on clinical samples. A series of calculations will be undertaken which are 'Type A' evaluation of uncertainty. Type A evaluations are used to estimate random errors arising from random variations of the test. A number of sources may contribute to the variability each time a measurement is taken which cannot be eliminated. The variability can be estimated by using statistical analysis of a series

of observations, and are used to obtain a value for both the repeatability and reproducibility of a process.

The repeatability of the procedure will be determined by using a single DNA and repeating the measurement 20 times within the same PCR. This will estimate the variation in the analysis which could be due to short-term temperature fluctuation across the PCR instrument in individual wells, or short-term current fluctuation across the ABI 3500XL. DNA originating from a blood sample is homogeneous and measurements on twenty aliquots from one single blood sample should allow the Laboratory to assess whether a difference in results contributes to the uncertainty of the measurement procedure.

The reproducibility of the technique will be determined by using a single DNA and repeating the measurement 20 times over 20 different PCR reactions carried out on different days, different operators and different thermal cyclers. This will estimate the variation in the analysis which could be due to different instruments being used for the PCR, variability in the performance of the individual carrying out the test and reagent changes such as polymer on the ABI 3500XL.

The calculation of the experimental standard deviation of the mean is a two-step process. The estimated standard deviation is calculated using the values which have been measured (% donor engraftment) – SOP IB008. The experimental standard deviation of the mean is then obtained by dividing this value by the square root of the number of measurements that contributed to the mean value.

The independent components are then squared and added together, and the square root is then taken. The resulting combined standard uncertainty is multiplied by a coverage factor k to provide the expanded Uncertainty, U. Accepted international practice is to use a coverage factor of k=2, this will give a coverage probability of approximately 95% assuming a normal distribution.

Results

The results from Table 2 indicate the results from the reproducibility of the procedure. This has encompassed factors which affect uncertainty – operator, thermal cycler, reagent changes on the ABI 3500XL. The results are shown as date of the test, the operator, thermal cycler used, mean % donor and estimated standard deviation. The standard uncertainty has been determined by using the following calculation:

$$U = s - \frac{\text{estimated standard deviation}}{\sqrt{n} = \text{number of measurements}}$$

Appendix B: Worked example

Table 2: Results from the reproducibility of the chimerism monitoring procedure, using the % mean from 20 different PCR assays

Date of test	Operator	Thermal cycler	Mean % donor	Divisor	Mean
17/04/2015	JC		84.6	20	
20/04/2015	JC		84.9	20	
22/04/2015	JC		85.2	20	
24/04/2015	JC		84.9	20	
28/4/2015	JC		83.5	20	
01/05/2015	JC		84.8	20	
06/05/2015	JC		86.0	20	
07/05/2015	BA		84.7	20	
11/05/2015	SM		84.7	20	
13/05/2015	JC		84.0	20	
15/05/2015	SM		84.3	20	
18/05/2015	SM		84.5	20	
20/05/2015	JC		84.0	20	
21/05/2015	SM		84.6	20	
26/05/2015	JC		84.6	20	
28/05/2015	SM		83.9	20	
29/05/2015	JC		84.7	20	
02/06/2015	JC		84.2	20	
04/06/2015	SM		83.9	20	
08/06/2015	JC		84.6	20	
Mean					**84.5**
Estimated standard deviation					**0.544929**
Standard uncertainty = u					**0.1218**

The results from Table 3 indicate the results from the repeatability of the procedure. The results are shown as date of the test, the operator, thermal cycler used, mean % donor and estimated standard deviation. The standard uncertainty has been determined by using the following calculation:

$$u = s - \frac{\text{estimated standard deviation}}{\sqrt{n}}$$

n = number of measurements

Table 3: Results from the repeatability of the chimerism monitoring procedure, using the % mean from 20 measurements within one PCR assay

Appendix B: Worked example

Date of test	Operator	Thermal cycler	Mean % donor	Divisor	Mean
16/06/2015	BA	15	85.0	20	
			84.5	20	
			84.5	20	
			84.2	20	
			84.4	20	
			84.8	20	
			84.2	20	
			84.4	20	
			85.0	20	
			84.9	20	
			83.8	20	
			85.2	20	
			84.4	20	
			84.4	20	
			84.8	20	
			84.5	20	
			83.8	20	
			84.2	20	
			84.9	20	
			83.6	20	
Mean			**84.5**		
Estimated standard deviation			**0.43**		
Standard uncertainty = u			**0.097**		
Combined standard uncertainty = U			**0.32**		

Appendix B: Worked example

The combined standard uncertainty **= ($\sqrt{0.1218^2 + 0.097^2}$) x coverage factor (2)**
= 0.16 x 2
= 0.3 rounded down.

The value of the measurand in % donor for the NEQAS IQA 152 DNA sample is 84.5% donor +- 0.3% donor.

"The reported expanded uncertainty is based on standard uncertainty multiplied by a coverage factor k=2, providing a coverage probability of approximately 95%. The uncertainty evaluation has been carried out in accordance with UKAS requirements. This measurement is derived from document M3003, *The Expression of Uncertainty and Confidence in Measurement (Edition 3*), November 2012 UKAS".