QUALITY CONTROL IN MOLECULAR MICROBIOLOGY

CONTAMINATION CONTROL

Contamination can occur at different stages:

- During **specimen collection or transport**
- During intitial **specimen handling** by the laboratory during procedures other than nucleic acid testing
- Contamination of specimens during **extraction**
- **Amplification**
- Product **detection**
- Contamination of **reagents**

Source of contamination can be:

- Contamination from **other** (positive) **samples**
- **Positive control** material
- **Amplified product**. Can occur with target or probe amplification methods, but not signal amplification assays.
- **Operator** who has the disease (eg: HSV)
- **Operator DNA** (eg: shed squames)

3 basic areas important for controlling contamination: staff competency, laboratory design and using controls to check for contamination.

Controlling contamination

- **Unidirectional workflow**. Each areas needs dedicated equipment and supplies.
    - **DNA free area** for reagent preparation to
    - **Specimen processing area**
    - **Amplifcation and detection area**
- Store reagents in **single-use** aliquots.
- Use 2-10% **sodium hypochlorite** to eliminate nucleic acid from work surfaces and equipment, followed by wiping down with 70% ethanol (to stop corrosion by the bleach). UV light can also be used to destroy DNA, but may need up to 8 hours, depending on the length of rhe fragments.
- **Careful handling of specimens** (training of staff to be competent important!)
    - avoid splashing
    - opening only one specimen tube at a time
    - pulse-spin tubes prior to opening
    - use screw-tops rather than flip-tops to minimise aerosolisation
    - and use plugged pipette tips
- Use positive control material at the lowest concentration that is consistently amplified.
- Use of **uracil-*N*-glucosylase (UNG)**. This enzyme inactivates DNA sequence that has dUTPs incorporated instead of dTTPs. If dUTPs are used in the PCR amplification, then it can be added to the reaction mixture prior to amplification where it will destroy any contaminating amplified product which contain dUTP, while not affecting template DNA which contains dTTPs. It is activated at 50ºC. Thus the subsequent PCR cycling must not fall below 55ºC to avoid destruction of amplified DNA by UNG. Likewise, at the end of the procedure, the mixture must be held at 72ºC. Doesn't work for RNA. May reduce efficiency of amplification. This is used in the Roche COBAS Amplicor system.
- Use of photochemical inactivation with **isopsoralen**. Here isopsoralen is added to the PCR mixture, and they intercalate into the newly synthesised DNA molecules. At the end of the PCR amplification, prior to opening the reaction tubes, long wave UV light crosslinks the isopsoralens to the DNA, rendering it refractory to further amplification and thus preventing it from becoming a contaminant. However, can be somewhat inhibitory to the PCR, and may affect probe hybridisation to some degree.

- Good **hygiene** – wear gown, gloves and change between different tasks
- Use **controls** to detect contamination

See nature article and NPAAC guidelines.

QUALITY CONTROL AND ASSURANCE

See review and NPAAC guidelines, AS 17025, JCM article

Three main aspects to quality assurance: evaluation of new tests, using control specimens, and participating in QAP.

Verification is the initial process whre sensitivity specificity and accuracy of a assay are established. **Validation** is the ongoing process that shows an assay is performing as expected. ?this is not what the review says….

Verificaton needs to be **analytical** – giving information on tha performance characteristics of the assay, as well as **clinical** giving information about the clinical utility of the assay. Can be difficult when the gold standard (culture) is less sensitive, giving the impression of being a non-specific assay. In this instance, also use clinical diagnosis as part of the gold standard, and verify the nature of the PCR product. Also difficult for rare pathogens, where adequate clinical samples are not available.

Validation (? Or verification) has a number of features:

(i) testing of dilution series of positive samples to determine the **limits of detection** of the assay and their linearity over concentrations to be measured in quantitative NAT;
(ii) establishing the day-to-day **variation** of the assay's performance
(iii) evaluating the **sensitivity and specificity** of the assay as far as practicable, along with the extent of cross-reactivity with other genomic material; and
(iv) assuring the quality of assembled assays using **quality control procedures** that monitor the performance of reagent batches before introducing new lots of reagent for testing.

Each time a new specimen time is introduced or there is modification of the procedure, the procedure needs to be re-validated. This includes re-validation all of the products in a multiplex procedure when one of the parts of the multiplex is adjusted. New batches of reagents should always be run in parallel with old batches to make sure results are as expected.

Need to keep maintenance records of equipment. Need to check in multi-well cyclers that the product produced on inner wells is the same as the amount produced on outer wells.

1. Requirements for validation:
   - 1.1. **Selection of primers and probes**: need to be from a highly conserved region and be specific for the target organism. Should annually check selected probes and primers against databases to check for cross-reactions
   - 1.2. **Confirmation of product**. During assay development, best achieved by sequencing. Alternatives are hybridisation probes, restriction enzymes or nested PCR. During day-to-day operation of the assay, can use melting curve analysis or simple molecular weight on a gel.
   - 1.3. **Standardisation of reagents and protocols**. Need to specify methods for preparation of reagents, and expiry dates. Any changes require revalidation.
   - 1.4. **Determination of a cutoff**. Run a dilution series to determine the limits of detection. Should range from undetectable to strongly positive. May need to run closer dilutions around the cutoff if precision is required. Need to do at least twice to confirm.
   - 1.5. **Between run variation**. Determined using a sample just above the cutoff and another sample of slightly higher concentration (less than 10 x), run multiple times (at least 20). These should also be used in the assay proper as positive controls.
   - 1.6. **Sensitivity and specificity**.
     - 1.6.1. **Specificity**. Need to test at least 100 negative samples. Samples are determined to be negative if the 'gold standard' is negative, there is no clinical evidence of disease and there is no detectable nucleic acid by another validated assay (eg: a commercial assay). If adequate specificity is not obtained (eg: >95%) redesigning the assay to improve specificity or using a second, confirmatory assay that does have a high specificity is

required. The second assay should target a different sequence. See example of *N. gonorrhoeae*.

1.6.2. **Sensitivitiy**. Should be tested with 100 positive samples. Samples are determined to be positive if the 'gold standard' is positive, there is clinical evidence of the disease and there is detectable nucleic acid by another validated assay (eg: a commercial assay). If 100 positive samples are not obtainable, then using testing using a matrix spiked with cloned or genomic nucleic acid of the organism (genomic is preferred) is acceptable. Should test in the presence of human genomic material. Standards are often commercially available (especially for viruses).

1.7. **Cross reactivity.** Should try to determine if likely commensual organisms will cross react by spiking negative samples with them. Examining sequence data on Genbank should provide the details of likely cross-reacting organisms.

1.8. **Validating quantitative assays.**

1.8.1. **Determine linearity.** Do a dilution series of a sample with known copy number (at least 5 dilutions, each run 4 times) and make sure it is linear over the clinically relevant range.

1.8.2. **Determine cutoff.** Here, use 3 samples – at, just below and just above the cutoff determined above. Preferably WHO reference samples are used. Each sample should be run 3 times on 8 consecutive days. The cutoff is the lowest concentration that is positive 95% of the time.

Positive controls

Designed to ensure the test can consistently detect a concentration of target nucleic acid near the limit of detection of the assay – want it at the lowest concentration that can be reproducibly amplified, both to enable detection of small increases in amplification efficiency, but also to avoid contaimination. Nature of positive control material depends on what is available – may use purified nucleic acid, or lysed or intact organisms. Using intact organisms allows for the control to be used as an extraction control that tests the success of the extraction or purification process. At ICPMR, use a 10-fold dilution series of the control, and plot the results to determine the cutoff with each run. It must be within 1 dilution of the mean for the result to be valid.

Monitoring for inhibitors

Important, especially for complex specimens such as blood or sputum. Inhibitors include (but are not limited to) haem, glycoproteins,. Heparin, phnol, urine crystals and EDTA. A number of methods are available:

- **Spiked samples**. Here each sample undergoes two amplifications, one directly, and one spiked with positive control DNA. The latter should be positive, or else the test shows inhibition. The concentration of the spiked DNA should be close to the limit of detection so that low level inhibition is detected.
- **Internal control**. This involves adding template nucleic acid with the same primer binding sites as the target, but with different intervening sequence to the sample prior to extraction. The different sequence allows separate detection of the resulting amplified product. If it is not detected, then inhibition has occurred. The Roche Cobas Amplicor has an internal control. In quantitative assays, this internal control can also be used as a quantitation standard.
- **Human housekeeping genes**. Such as β-globin or DQA can be amplified as internal controls, but if it is there in amounts much greater than the target, inhibition of the target amplification may occur without noticible inhibitoion of the housekeeping gene.

Always need to monitor for inhibitors when a new specimen extraction method or specimen type is used. However, for cost reasons, may discontinue these when the inhibition rate is found to be < 1-2%.

Can try and remove inhibitors with a freeze-thaw cycle. Also by testing specimen in a 1:100 dilution, which dilutes inhibitors, but hopefully will not dilute DNA below the level of detection (also test the specimen neat).

Sometimes want to make sure there is adequate DNA in a specimen to start with – eg: with paraffin embedded tissue where formalin fixation cross-links protein to nucleic acid that may prevent subsequent amplification. Can amplify human housekeeping genes to check.

Negative controls

Need to include in all assays, and process in a manner similar to clinical specimens. Need to take negative controls through all steps of the assay, including extraction. A negative result for the negative control does not exclude contamination, as contamination can be low level and sporadic. Ideally the negative control is a clinical specimen known not to contain the target DNA, but this may be difficult to obtain, and water or buffer may be substituted. At ICPMR, often use water as the negative control or no DNA control (NDC). An NDC is included from the start of specimen processing for each specimen (the specimen control), and when the PCR assay is setup, a second NDC control is used (the run control) for each specimen – inserted between each specimen.

Proficiency testing
Should participate in available QAP.