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Practice Guidelines for the Use of the WAVE System in Diagnostic Service

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1.0 INTRODUCTION

These best practice guidelines were updated following open discussion at the end of the 3rd WAVE diagnostics users meeting (16-17 October 2003, NGRL (Wessex)). The majority of laboratories in the UK were represented, as well as several centres from Belgium and the Netherlands and Germany. All participants agreed that the guidelines should primarily be practical and workable in their application to use of the WAVE (Transgenomics Limited, Crewe, UK) in diagnostic service. These guidelines assume adherence to existing manufacturers' instructions.

The guidelines have been divided into recommendations to ensure effective quality assurance of the laboratory process, use of software, data handling and optimization of the dHPLC system.

2.0 QUALITY ASPECTS OF THE LABORATORY PROCESS

2.1 Template concentration

In order to achieve consistent WAVE traces, it is strongly recommended that the amount of template DNA be limited to 10-100 ng.

DNA extracted with some commercial systems may contain impurities that negatively influence PCR. Diluting to 10 ng may overcome these effects.

2.2 PCR buffer mix

The PCR must be oil-free. Certain additives in polymerase buffers can have an adverse effect on the performance of the DNASep column. A list of polymerases and buffer systems recommended for the WAVE can be obtained from Transgenomics (Salisbury polymerase preferences: Optimase, AmpliTaqGold, HotStarTaq). Users are free to validate other buffer systems/polymerases etc, but should be aware that the column warranty relating to the number of injections could be affected. Some groups repor1ted spiking Optimase with non-proof reading Taq polymerases can overcome problems of optimization e.g. 0.05 u Platinum/1 u Optimase.

2.3 Heteroduplex formation

The following temperature parameters are recommended for efficient heteroduplex formation. This can be set up as a PCR cycle as follows (e.g. on MWG Primus thermocycler):

95 ºC 5 min

95 °C for 22 seconds; 69 cycles: (T-1 °C) 22 seconds.

Where T = temperature of previous cycle

NB *Some published faster cooling protocols used for heteroduplex formation can seriously impair heteroduplexing. (Els Scholen, unpublished data)*

Following formation, heteroduplexes should either be run on the WAVE immediately or stored at -20 °C until required. Heteroduplexing just prior to a run and after long term storage (greater than 2 weeks), is preferred by some groups although there is no firm evidence that this is beneficial. It is not clear how stable heteroduplexes are a –20 or 4°C.

2.4 Post PCR processing

This should not be necessary prior to dHPLC.

2.5 PCR product check

For autosomal recessive, X-linked recessive and X linked conditions for males, PCR products from normal and patient samples should be mixed in equal proportions. It is recommended that PCR products are run on an agarose gel to check for efficient amplification of both the normal and patient samples. It is particularly important that the intensities of the fragment bands are relatively uniform. For instance, if the band from the normal control is relatively weak compared to the patient samples, any mutations present may not be detected due to inefficient heteroduplex formation.

For autosomal dominant conditions (and X-linked dominant conditions in females), checking the amplification efficiency prior to heteroduplex formation is purely a personal preference.

2.6 Controls

Positive controls: If available, a mutation-positive as well as a confirmed normal DNA control should be included for each gene fragment amplified and subsequently screened on the WAVE. When a mutation control is unavailable, a confirmed normal control must be included. When amplifying large numbers of samples for many different gene fragments with a low frequency mutation pickup rate, it is sometimes acceptable to omit a normal control.

Negative controls: Negative controls containing no template DNA must be included for each amplicon (one per amplicon per plate), to check for contamination. Contamination can be excluded by agarose gel electrophoresis or WAVE analysis. It is not necessary to run negative controls on the WAVE. Asymmetric positioning of controls can help with plate orientation.

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3.0 SOFTWARE, ASSESSMENT OF CHROMATOGRAM QUALITY AND IDENTIFICATION OF SEQUENCE VARIANTS

3.1 Current software

At present, it is recommended that the melting profile of DNA fragments should be predicted using Navigator, WAVEMAKER 4.1 algorithms (or later versions), which will ensure the most accurate estimation of melting. It is important to ensure the whole sequence (including clamps if used) is included in the melt analysis otherwise gradient mispredictions will occur. 'Navigator' software (Transgenomics Ltd, Crewe UK) is based on ORACLE, it is more versatile and allows for 'normalization' of peak heights. This system should hopefully be less prone to mid-run failure than existing softwares. ***NB** In our experience switching the computer off and on prior to starting a new run can alleviate some of the failure problems (Rachel Wycherley personal comm.). It is advisable to print out any error messages prior to switch off. These will be useful to the Transgenomic helpdesk 'sick' machine diagnosis*
Predictions made with WAVEMAKER and Navigator tend to be less accurate (generally too low) for the GC rich, high temperature (>59 ºC) melt domains of amplicons. Empirical melt evaluation of these amplicons is advisable.

3.2 Minimum peak intensity

Ideally the signal intensity of dHPLC profiles should be higher than 2 mV at A_{260} [Ref 1]. Peaks of intensity less than 1 mV should be rejected and either the injection repeated, or the sample amplified again and reinjected. Such weak peaks are more likely to lead to false-negative/positive results. Furthermore, changes in retention time do not accurately predict the presence of a sequence change. Instead, changes in peak profile should be used to predict the presence of base changes.
The minimum peak intensity depends on the 'normal' peak intensity of the run. Peaks with intensities less than ~30% of the 'normal' should be viewed with caution. This is especially true when using the normalization tool in 'Navigator'. Small aberrations can be exaggerated creating false positives
***NB** Automated systems for equalizing template concentration prior to PCR are available and would be a desirable option.*

3.3 Visual assessment of chromatograms

Overlaying a number of chromatogram traces is a useful tool; however, differences in template concentrations are often reflected in the different peak heights and unless peaks are exactly equivalent or 'normalized', subtle changes in peak profiles can easily be missed. It is therefore recommended that peaks are first viewed singly, possibly in a tiered block including a 'normal' trace.

3.4 Trace specificity

Trace profiles are not unique for a specific mutation, i.e. different mutations and polymorphisms can give identical profiles (see 4.2). It is therefore strongly recommended that sequencing [Ref 2] or an equivalent assay designed specifically for a known mutation e.g. ARMS, should be used to verify the presence of a mutation in a patient or family. It is usually sufficient to report the absence of a familial mutation (which is clearly detected by dHPLC) on the dHPLC evidence alone.

4.0 DATA CHECKING, REPORTING AND STORAGE

4.1 Data checking

Two of the approaches currently in use include:
4.1.1 All peak profiles are printed out for checking. This method is suitable for gene analysis that only includes a small number of amplicons, e.g. Rett syndrome, *MECP2* gene, 8 amplicons (David Bunyan personal comm.).
4.1.2 Only abnormal peak profiles/chromatograms are printed out together with a positive and normal control for checking. All profiles are scored (as normal/abnormal) by the operator on to a printed worksheet. The checker then checks the chromatograms 'on screen' against the printed worksheet, which is then initialed and dated. This method is suitable for gene analysis that includes a large number of amplicons and helps to reduce the paper load, e.g. Marfan syndrome, *FBN1* gene, 65 amplicons (Rachel Wycherley personal comm.).

4.2 Positive results

Abnormal chromatogram traces should be investigated by sequencing. It is recommended that for the sequencing, a new PCR reaction is prepared using a fresh template from the patient sample. This should eliminate the possibility of detecting PCR artifacts giving rise to the abnormal chromatograms.
An alternative to sequencing, when a common polymorphism is encountered, is to mix the test DNA sample with a sample heterozygous for this polymorphism and then carry out heteroduplex and dHPLC. No apparent change in the profile after the addition of the known polymorphic DNA would suggest that no other mutation is present in the test DNA (Peter Davis/Sian Ellard personal comm.).

4.3 False positive results

These are minimized if a positive mutation is taken to be a d efinite change in pattern from a reference sample as opposed to multiple peaks for a mutation compared with a single peak pattern for a wild type. A multiple peak pattern can be easily produced by combination of poor fidelity polymerase and high cycle number PCR.

4.4 Negative results

Negative results should be reported as 'no mutation detected', stating the gene or portions of the gene screened, the percentage of known mutations eliminated (if known) and the detection method used.

4.5 False negative results

Failure to detect mutations may arise for a number of reasons.
Provided the mutation standards are working correctly the most likely problems are due to:
(i) Poor quality PCR. Very low yields can affect the ability to see mutations. Very high yields can lead to large proportions of misincorporations and hence increased difficulty in calling mutations. These problems can also be due to variation in the fidelity of PCR enzymes.
(ii) Mutations that occur in a high melt GC-rich pocket in a fragment with otherwise normal nucleotide ratios. In order to resolve these, it may be necessary to redesign fragments, preferably with the GC-rich regions towards the ends of the fragments. GC clamps may also be used [Ref 3].

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(iii) The small size of the fragment. Mutations in fragments less than 110-120 base pairs can remain undetected unless some method of fragment extension is used.

4.6 Sensitivity

Claims regarding the sensitivity of dHPLC vary but largely reflect the committed expertise of the users and care taken in designing the analysis and the sequence examined, e.g. position of CG-rich regions and size of fragment (usually 150 bp-450 bp), and number of injection temperatures used. With these points in mind the general consensus is that, at best, dHPLC is a method with high (>95%) sensitivity and comparable to direct sequencing [Ref 4]. Increasing the fragment length screened, e.g. >500 bp, invariably results in the need to accommodate more melt domains and hence more injections per sample at a range of oven temperatures. Generally the use of larger fragments results in a lower sensitivity independent of the number of injection temperatures and reduction to smaller fragment sizes (>120bp see 4.5.3) is recommended.

4.7 Detection of mosaics

Mutations can be detected at very low levels using dHPLC. This is useful for mitochondrial analysis, tumor samples and somatic mosaics. The difficulty occurs when a pattern change has been seen resulting from a low level mutation which is not able to be verified by sequencing due to the fact that sequencing does not generally call mutations when present at less than 30%. If there is a definite and reproducible change that cannot be verified by direct sequencing of the DNA, then enriching the DNA for mutant peaks using a fragment collector followed either directly by sequencing or by cloning and sequencing will enable location of the mutation.

NB *Collection can be done without a fragment collector by manual collection and careful timing.*

4.8 Archiving

Monthly applications may be transferred to CDs, optical discs or even Zips. Jaz drives have been used but as these may soon become obsolete, transfer to CD or optical disc is recomm ended. Many PCs now include CD rewriters and as CDs or even DVDs are relatively cheap they may be the preferred option. For backup, duplicating (CDs or alternative) is a good habit with one as a working copy and one as a storage backup. Backup servers for up to four systems are a good alternative to network servers.

5.0 DHPLC SYSTEM GUIDE LINES

5.1 Buffers

Only TEAA supplied by Transgenomics should be used to make up buffers A and B. In addition, HPLC-grade acetonitrile and 18 megOhm water are also strongly recommended. Buffers should be made up on a weekly basis or more frequently if required.

NB *We are currently getting consistently good results using ready-made buffer solutions now available from Transgenomics. The costs are a bit higher but these have proved useful for convenience and removal of another potential variable. However, batch to batch variations have been reported, causing changes in peak retention times. Each new batch should be evaluated in house.*

5.2 Instrument controls

Fragment size standard (pUC18/*HaeIII*) and Mutation Detection control (DYS271) function as instrument controls. These controls should be run through the system as a matter of course whenever the buffers are changed. In addition, controls should also be run after any system change, e.g. changing DNASep column, in-line filter etc, otherwise an instrument problem or a loss of resolution on the heteroduplex resolution on the cartridge may not be obvious during the analysis or may go unnoticed. Results from the controls as well as system performance should be recorded in a log-book on a regular basis.

5.3 Column pressure

It is good practice to monitor the column pressure on a regular basis. If the pressure increases at a flow-rate of 0.05/0.9 or 1.5 ml/min, it is recommended that the column is cleaned with 100% buffer C at a flow-rate of 0.9 ml/min and a temperature of 80 ºC for 15 minutes. The temperature should be reduced to 50 °C and the column equilibrated with 50:50 A and B for at least 1 hour. HT systems may be cleaned at 0.9 or 1.5 ml/min.

5.4 Needle injection port syringe checks

Prior to running samples through the system, it is recommended that the needle and injection port is washed 10-20 times. The syringe should be inspected on a regular basis to check for signs of leakage and damage to the plunger seal. In general, the life of the syringe is usually around 6 months or 1000 injections.

5.5 Foil and film use

Recommended options include the ABI septa or Transmat Silicone Sealing Mats from Transgenomics. Details of suitable foil/film covers can be obtained from Transgenomics.

6.0 REFERENCES

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