



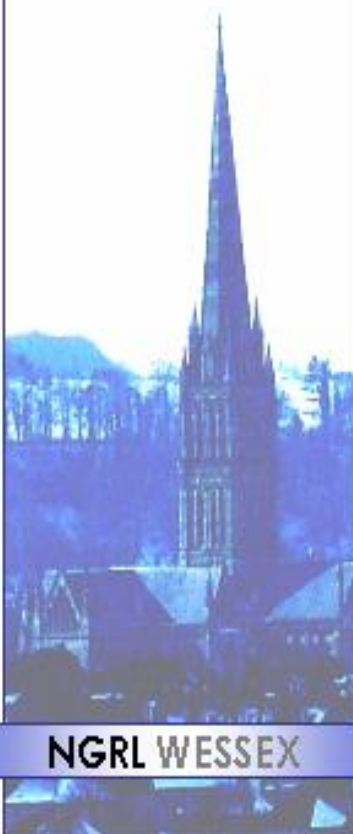
National Genetics Reference Laboratory (Wessex)



NHS


Application Note

Standardised Primer Optimisation and Design Specification



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1. INTRODUCTION

Effective use of automated liquid handling for setting up simple PCR based mutation scanning and detection invariably requires primers to be designed to work at standard annealing temperature. This allows different fragments, either from the same gene or different genes, to be batched together on the same micro titre plate as required. Consequently, with the introduction of automation, many laboratories undertake complete redesign of primer sets for their genes of interest. This design and optimisation process can be relatively time consuming and may often be replicated for a particular gene in different laboratories.

This document details a Standardised Primer Optimisation and Design Specification (SPODS) that includes parameters relating to choice of fragments, reaction components, amplification conditions, optimisation and validation. This specification is intended to serve three functions:

1. To describe a general system of PCR that allows standardisation of primer sets both within a laboratory and between laboratories.
2. To describe and detail the use of currently existing primer sets designed in this laboratory so that other laboratories can successfully implement their use.
3. To provide a basis from which other laboratories can design similar primer sets that are compatible and therefore usable in any laboratory where the system is already in use.

Use of this specification in different laboratories will have a broad range of benefits including:

- Avoiding duplication of effort
- Equity / comparability of testing between laboratories
- Transportability of assays between laboratories
- A framework for documentation for accreditation (e.g. IVD)
- Compatibility with automation
- Opportunity for centrally administered repository of primer sets including feedback on performance issues.
- Compatibility with gene specific reference control mixes available from NGRL(W)

Ideally all aspects of the specification should be adhered to as this will yield primers that have the most universal application both in terms of methodologies and inter-laboratory exchange. However, in the short term, it may be preferable to move towards this type of universal standardisation in a stepwise manner. In these cases it would be beneficial if annealing temperature and the tag sequences used were prioritised for standardisation.

Since different analysis methods may have different specific design requirements, this specification has been principally developed to accommodate the two major PCR based applications currently in use: direct sequencing and analysis of fluorescently labelled fragments. However this does not exclude other applications and input regarding specification parameters and values would be gratefully received.

2. CENTRAL REPOSITORY OF OPTIMISED PRIMER SETS

There are a number of primer sets currently designed and optimised using this system. These are detailed in section 13 below and are available on request from National Genetics Reference Laboratory (Wessex) – please contact chris.mattocks@salisbury.nhs.uk. Users of these primer sets are encouraged to feed back any problems or modifications so that these can be logged and passed on to other users. In addition anyone using this system to design new primer sets is encouraged to submit their designs for inclusion in our database.

3. SYSTEM OUTLINE

The system is designed to produce primer pairs that all perform under a standard set of amplification conditions and have 5' universal tags. A single pair of universal tags designated US1 and US2 (see section 4.2 below) are used. Each primer comprises a gene specific (GS) sequence with either US1 or US2 tag sequence appended to the 5' end. Some primers also have filler bases[s] between the GS sequence and the tag: these are included to prevent the formation of a highly stable hairpin structures. Primers with the US1 tag are designated GS1 and primers with the US2 tag are designated GS2. The final component of the system is a set of universal primers, which are simply the universal tag sequences US1 and US2. These can be labelled or tagged to suit a wide range of applications.

Each primer pair comprises one GS1 primer and one GS2 primer. There is no correlation between the orientation of the GS primer and the tag that is attached to it (i.e. a GS1 primer could be either a forward or reverse primer with respect to the gene orientation). With respect to sequencing, it is therefore important to be aware of the orientation of the sequence that will be generated using a particular universal primer with a particular amplicon.

The tagged primers can be used in three basic formats. Most simply in the **two primer system**, tagged primer pairs are used in a standard PCR to produce amplicons that are differentially tagged at either end (fig. 1). These amplicons can be independently sequenced in either orientation using either US1 or US2 as the sequencing primer. This system is widely used to facilitate the automation of sequencing pipelines as it allows any amplicon to be sequenced in both orientations using just two master mixes.

Similarly, with **two round labelling**, tagged amplicons are generated in a standard PCR. These products can then be used as the template for a second round of PCR in which US1 and US2 are used as the primers. However, in this case, one or both of the US primers is labelled or tagged (e.g. US1-FAM and US2-VIC) in order to produce a suitably modified amplicon (fig. 1). This allows any amplicon to be suitably modified at will. This system is most useful where the amplicons are required to be modified at both ends, for example for fluorescent SSCP.

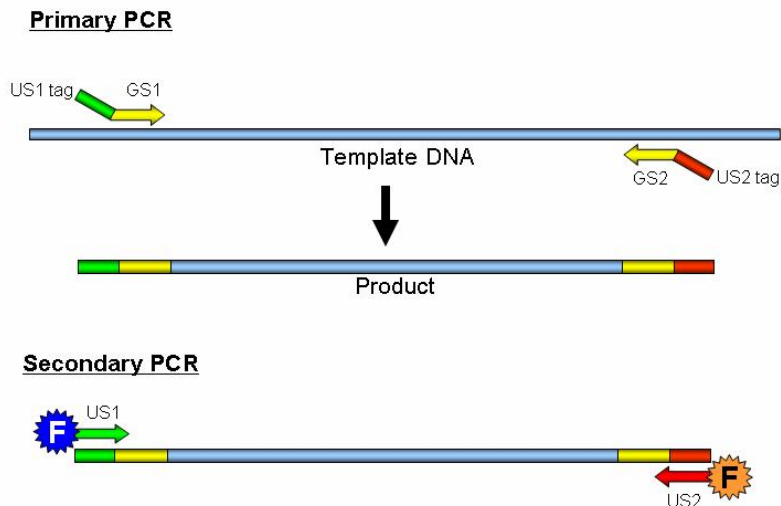


Figure 1: In the two primer system fragments are amplified under standard PCR conditions using two gene specific primers: a GS1 primer that has a US1 tail (green) and a GS2 primer with a US2 tail (red). Consequently both strands of the PCR products are tagged: one orientation having a US1 tag and the other having a US2 tag. These products can be independently sequenced in either orientation using either US1 or US2 as the sequencing primer. Alternatively, with two round labelling, the product of the primary PCR can be used as the template for a secondary PCR using suitably labelled US primers facilitating independent labelling of both strands of the product.

Where a single modification to an amplicon is required a novel **three primer system** can be used. Here the PCR contains the GS1, GS2 and a modified US1 primer (e.g. US1-FAM). The GS1 primer is used at a limiting concentration so that it effectively depletes during amplification allowing the labelled US1 primer to take over. This facilitates amplicon specific modification to be carried out in a single PCR. In addition, since the amount of labelled product is a direct consequence of the amount of modified US1 primer that is used, fluorescent yield can be relatively easily controlled and normalised between different reactions by controlling the quantity of US1 used. This system has been successfully used for conformation sensitive capillary electrophoresis (CSCE), where different fragments can be labelled in different colours to allow product mixing for parallel analysis on a capillary instrument.

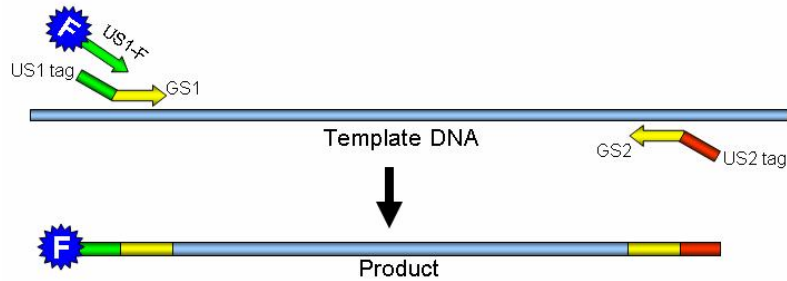


Figure 2: In the three primer system a modified US1 primer (US1-F: fluorescent) is used in addition to the GS1 and GS2 primers. The GS1 primer is used at limiting concentration so that it depletes during the early PCR cycles and amplification is taken over by the US1 primer thus labelling the product in a single PCR reaction.

Whilst this system has been designed to produce tagged primers, the gene specific sequences (i.e. the 3' sequences in each primer that are complementary to the template) can obviously be used in isolation if required. Since the tagged primers provide so much more flexibility and the tags will not generally be detrimental to down stream processes even if they are not required, this strategy is not recommended. However, a brief description is given in section 11 below.

4. DESIGN SPECIFICATION

The following specification has been followed as far as possible for all primer sets designed in this laboratory. It should be noted that much of the specification is designed to simplify the design of primers and in some cases it has been necessary to disregard one or more parameters in order to obtain a working primer pair. However the key parameters, referencing, tag sequences and annealing temperature have been preserved in all cases.

4.1 Primer Referencing

All primers have been referenced using the following system

a
b
c
d
e
 BC1_11C_fwd_tag1_01

- a) Gene designation (BC1 = BRCA1 in example)
- b) Fragment designation (11C = exon 11 fragment C since exon 11 is covered by 12 fragments, A to L). In cases where an amplicon covers more than one exon the number of the first exon within the amplicon is used for naming purposes only (e.g. BC2_5 covers BRCA2 exons 5 and 6).
- c) Orientation of primer with respect to gene orientation:
 fwd = forward primer, binding site located on the anti-sense strand.
 rvs = reverse primer, binding site located on the sense strand.
- d) Designation of the 17bp tag attached to the 5' end on the primer; either US1 or US2.
- e) This defines the current version of the primer (01 = 1st design 02 = 2nd etc.). This is important when primers have to be re-designed since the new primer will be designed to a new binding site and will therefore have a completely different sequence.

4.2 Tag sequences and Universal primers

The universal primers are UniSeq (or N13) sequences. Since either tag can be used on either the forward or reverse primer, the tags have been designated US1 and US2 to avoid confusion over the orientation of sequencing reactions derived from their use. Each primer pair should have one primer with a US1 tag (GS1) and one primer with a US2 tag (GS2). The tag sequences are:

US1 = 5' – GTA GCG CGA CGG CCA GT – 3'

US2 = 5' – CAG GGC GCA GCG ATG AC – 3'

The use of universal primers allows any fragment to be labelled with any dye or tag at a substantially reduced cost (GS primers do not need to be labelled) and, with modification of the design, for many different purposes. A set of six universal primers should be adequate for most fluorescent applications (see table 1). It is also possible to add sequence tags to one or both universal primers if required.

Primer	Label	Use
US1_FAM	FAM	Fluorescent PCR
US1_VIC	VIC	Fluorescent PCR
US1_NED	NED	Fluorescent PCR
US1_PET	ROX	Fluorescent PCR
US1_U	Unlabelled	Standard PCR, sequencing
US2_U	Unlabelled	Standard PCR, sequencing

Table 1: Basic panel of universal primers for fluorescent labelling

4.3 Amplicon Length

In order to suit as wide a range of applications as possible amplicons have been designed to be between 250 and 500bp.

4.4 Buffers and analysable sequence

A 50bp 'buffer', including primer sequence, has been incorporated at either end of each amplicon. The buffer is defined as a region that can be ignored for the purposes of analysis. Such buffers are designed to facilitate methodologies, such as heteroduplex analysis, where the sensitivity drops off toward the end of an amplicon. All sequence between the two buffers is referred to as the 'analysable sequence'. In cases where a series of overlapping amplicons is required to cover a particular region the buffers should be mutually exclusive i.e. the whole region should be covered by analysable sequence in at least one of the overlapping amplicons.

It is important to note the effect of tag sequences in the context of analysable sequence. Since buffers include primer sequences, primers with tags will constitute most if not all of a 50bp buffer. Therefore use of primers without the tags as designed may significantly reduce the analysable sequence for an amplicon.

Analysable sequence containing an exon should extend at least 20bp into the intronic sequence.

4.5 Primer design

The actual programme used to design primers is not particularly critical, but a systematic approach is helpful to prevent high failure rates during optimisation. The vast majority of primers used in this laboratory have been designed using Oligo 5. This programme has been found to specify a large majority of primer pairs that work on first optimisation. In particular the ability to try different tags on the ends of primers and determine their effect on hairpin formation has been found to eliminate a significant number of primer pairs that are likely to fail wet testing. The parameters used with Oligo 5 are detailed in table 2 below.

Parameter	Setting
The listed items should be checked in the 'Search for Primers and Probes' dialogue box.	+ Strand search - Strand Search Compatible Pairs Duplex-free Oligonucleotides Highly Specific Oligos (3' - end Stability) Eliminate False Priming Oligonucleotides Oligonucleotides within Selected Stability Limits Hairpin-free Oligonucleotides Eliminate Homooligomers / Sequence Repeats
Minimum acceptable stringency	Moderate
Most stable 3' dimer	$\Delta G = -6.0$ kcal/mol
Most stable overall dimer	$\Delta G = -10.0$ kcal/mol
Most stable hairpin	$T_m = 40^\circ\text{C}$
Lowest acceptable maximum annealing temperature	68°C

Table 2: Primer Design Using Oligo 5

4.6 Database information

Various databases have been used to exclude polymorphisms from primer binding sites. On occasion, where this is problematic, degeneracy has been introduced into the primer to cope with the polymorphism. In general SNPs have been sourced from dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) but where gene specific databases are available these have also been used. In addition, local data from experience using different primer sets has been used where possible.

No attempt has been made to exclude mutations from primers in an overlapping sequence of amplicons. Where there is doubt about the significance of a variation it has been treated as if it is a mutation. It is therefore theoretically possible to miss a mutation because it lies in a primer binding site and is linked to a polymorphism in a binding site of an adjacent amplicon although the risk is very small. The HGMD database is a good source of information regarding mutations in many genes (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd/search.html>)

All primers have been subjected to a BLAST search to eliminate the pairs that could generate amplicons from sequence other than the specific gene required. Several sites have been used for batch BLASTing, the most useful of which are:

<http://greengene.uml.edu>

<http://www.ensembl.org/Multi/blastview>

BLAST searches have been performed using Blastn on nt database with default parameters.

4.7 Validation

Primer pairs have been validated using both two and three primer systems. Successful amplification of the correct sized product has been checked by running products on 2% agarose gels and fluorescent products using a AB 3730. Amplicons where non specific peaks greater than 10% of the main peak consistently appear above the 100bp marker have been either re-optimised or redesigned.

With respect to fluorescent analysis under partially denaturing conditions attention has also been paid to the quality of the peak. Where possible only clean / single (no stutters), sharp / narrow peaks (<500 scans on AB 3730 from base line to base line) have been used. In some cases it has not been possible to design out stutters. These appear to be a consequence of repetitive sequence or very high AT rich regions at the end of amplicons. Where this is the case the primer balance giving the least consequential stutter has been used (see section 10.2 below).

All amplicons have been sequenced in both orientations to validate them for sequencing and to ensure the correct reference sequence is generated. The use of PHRED scores to determine a threshold quality for sequencing is under investigation.

5. SPREAD SHEET INFORMATION

Information regarding primers in disseminated primer sets is given in the 'primers' worksheet of the Excel workbook supplied. The 'dilution schedule' contains information regarding primer concentrations required for the three primer system. A pictorial representation of the primers in the context of the gene is given in the 'seq' worksheet.

5.1 'Primers' worksheet

Each column of the table has a descriptive comment that can be viewed by mousing over the column header.

Primer sequences are given in excel spreadsheet format to facilitate electronic manipulation. Tagged primers comprise a genomic binding sequence (capitalised: green = forward primer, red = reverse primer), one of the two tags (lower case: green = US1, red = US2), and any filler bases inserted between them (capitalised, blue). Untagged primers represent the genomic binding sequence (GS sequence) only. In rare cases certain bases within the genomic binding sequence have been made degenerate to accommodate polymorphisms where they cannot be avoided. In such cases the IUB codes have been used to designate the mixed bases (table 3).

IUB code	R	Y	S	W	K	M	B	D	H	V	N
Bases	AG	CT	GC	AT	GT	AC	CGT	AGT	ACT	ACG	ACGT

Table 3: International Union of Biochemistry codes for mixed or degenerate bases

Reference sequences (i.e. amplified templates) have been given starting from the 5' end of the GS sequence of the forward primer through to the 5' end GS sequence of the reverse primer (i.e. excluding tag sequences). Locations have been given with respect to cDNA nucleotide numbering, which is defined according to the relevant reference sequence for each gene. Base 1 is defined as the A of the ATG start codon in the given gene. For intronic locations an indication of the distance from the nearest exon has been given (e.g. if exon 2 comprises nucleotides **101** to **199** inclusive, **101-155** is in IVS 1, 155 bases upstream of exon 2 and **199+98** is in IVS 2, 98 bases downstream of exon 2).

5.2 'Dilution schedule' worksheet

This spread sheet gives details of primer concentrations for use with the three primer system. The use of this sheet is described in detail in section 10.2 below.

5.3 'Seq' worksheet

The 'seq' worksheet contains a browser view of the gene of interest with all exons, coding sequence, translation and primers marked. Several conventions have been used to make this view simple to interpret:

- Non-exonic sequence is in lower case and coloured grey.
- Exonic sequence is in uppercase:
- Non-coding exonic sequence (5' and 3' UTR) is coloured black.
- Coding sequence is coloured blue and red alternately indicating codons.

Figure 3 below describes the browser features in more detail.

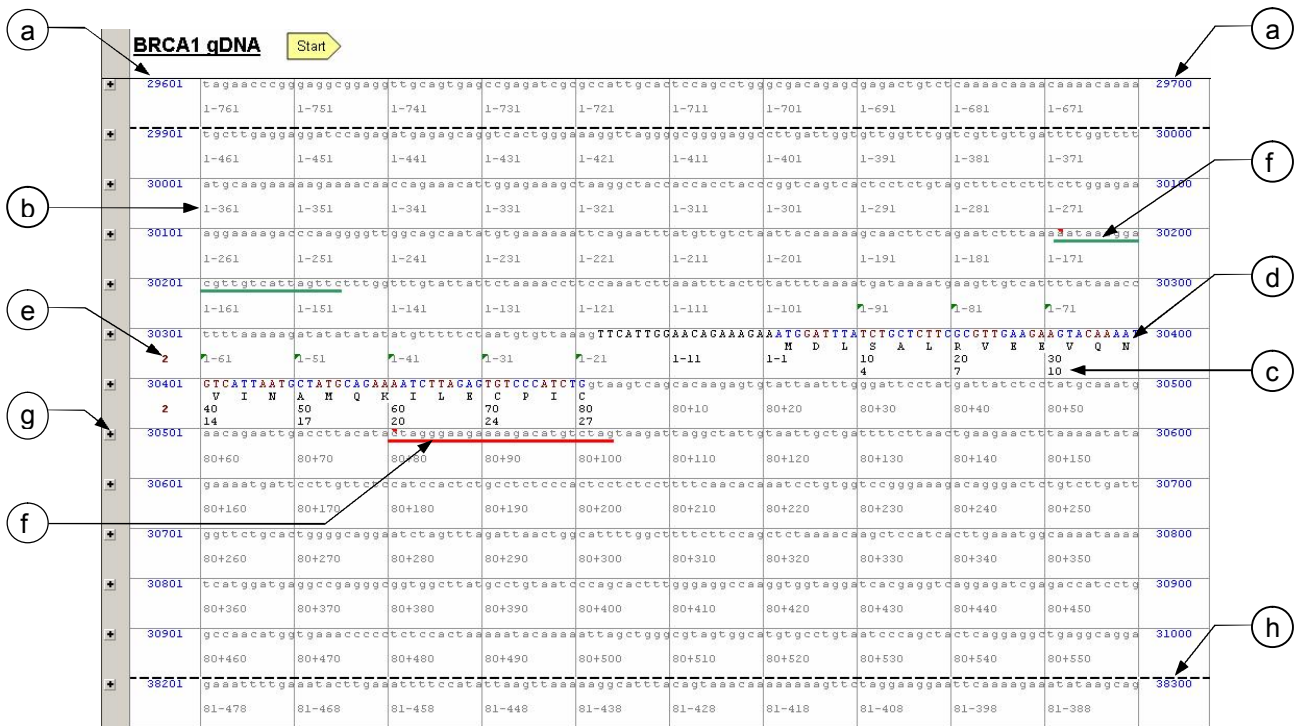


Figure 3: Details of the browser view contained on the 'seq' worksheet.

- Contig base number
- Nucleotide number: A of ATG = 1, non-coding bases numbered as '+' or '-' from nearest exon. Numbers given relate to the base immediately to the right of the vertical grey lines.
- Codon numbering. Numbers given relate to the base immediately to the right of the vertical grey lines.
- Amino acid translation (single letter code). Letters are positioned under the second base of each codon. Therefore, where a codon spans two exons, the translation is only labelled on the exon containing base two.
- Exon number: labelled when an exon is contained in a line.
- Primers: forward=green, reverse=red. Mousing over the red triangle on the left most end of the primer reveals a comment with the primer designation.
- '+' button indicates that some rows on the spreadsheet are hidden. Where the button is next to a grey horizontal line the hidden lines contain additional features – pseudogenes, repeat sequences, transcription factors etc. Each feature is identified in a comment located under the red triangle in the left most end of the feature. Where the button is next to a black dotted line (see h) pressing it will reveal intervening sequence together with all the associated features.
- Heavy black dotted line indicates that a portion of the sequence is hidden (this can be seen by comparing the blue contig base numbers above and below the dotted line). This sequence is hidden to provide a more convenient view of the coding sequence and flanking intronic sequences.

6. PRIMER ORDERING

It is recommended that primer orders are completed by using the spreadsheets provided: the use of the cut and paste function will eliminate errors in transcription. It is recommended that GS primers are reverse phase HPLC purified with MALDI_TOF quality check to ensure the absence on n-1 primers.

It should be noted that the synthesis yield for any given scale will decrease as the length of the oligo being synthesised increases. Since the GS primers are all approximately 40bp long a lower yield should be expected than for normal primers (18-22bp long) If the three primer system is to be used this will not necessarily be an issue since the GS primers are used at a very low concentration. However if the system is to be used for sequencing it is recommended that one synthesis scale greater than normal is used.

7. TWO PRIMER SYSTEM

This system is simply a standard PCR with the addition of universal tags to either end of the PCR product. The two main uses for this system are sequencing and two round labelling both described below (sections 8 and 9 respectively).

7.1 PCR setup

PCRs are normally setup in 10 μ l volumes although this can be scaled if required. For simplicity a 5x working stock containing 1 pmol/ μ l of both GS primers can be made for each amplicon.

Reagent	Final concentration		Quantity (μ l)
Template DNA (20 ng/ μ l)*	4	ng/ μ l	2
5x GS primer mix (1 pmol/ μ l each)	1	x	2
2x Amplitaq gold Mastermix (Applied Biosystems PN4326719)	1	x	5
H ₂ O			1

Table 4: Standard 10 μ l PCR setup for two primer system.

*In most cases 40ng will be suitable although varying the DNA concentration can help with problematic samples.

Because all amplicons designed using this system are universally labelled, the risk of cross contamination is potentially increased. However careful use of normal precautions including spatial separation of pre and post PCR workflows with dedicated pipettes for each and the use of filter tips should be adequate protection in most cases.

7.2 Thermal Cycling

All the primer sets have been tested and optimised on a MJ research PTC-225 DNA Engine Tetrad. Standard thermal cycling conditions are given in table 4.

Step	$^{\circ}$ C	Time		Cycle
Taq activation	95	10	min	
Amplification				} X30 cycles
Denature	95	5	sec	
Anneal	61	30	sec	
Extend	72	1	min	
Final extension	72	5	min	
Hold	15	∞		

Table 5: Standard thermal cycling conditions for the two primer system.

8. SEQUENCING

PCR products from the two primer system can be simply sequenced in either direction with appropriate choice of US primer for the sequencing reaction. All fragments can be sequenced in both orientations using just two sequencing reaction mixes; one with US1 as the primer and the second with US2.

Sequencing procedures vary from lab to lab according to instrumentation, chemistry, cleanup methodology, and availability of automation. The following procedure is currently optimised in this laboratory and is given as a guide only. This procedure is fully automated on a Perkin Elmer MultiProbe II robot, PCR machines are MJ research PTC-225 DNA Engine Tetrads, and the products are run on an AB3130xl. PCR and sequencing cleanups are performed using 384 well Millipore MultiScreen cleanup plates (Cat. Nos: S384PCR10 and S384SEQ10 respectively).

Reagent	Quantity (µl)
Template DNA (1:8 dilution of cleaned PCR product)	2.00
US1 <u>or</u> US2 primer (1 pmol/µl)	2.00
BigDye V1.1 (Applied Biosystems PN4336791)	0.25
5x sequencing buffer (Applied Biosystems PN4339843)	1.75
H ₂ O	4.00

Table 6: Standard 10µl sequencing reaction setup.

Step	°C	Time	Cycle
Amplification			
Denature	95	30 sec	} X25 cycles
Anneal	50	15 sec	
Extend	60	2 min	
Hold	15	∞	

Table 7: Thermal cycling conditions standard sequencing reaction.

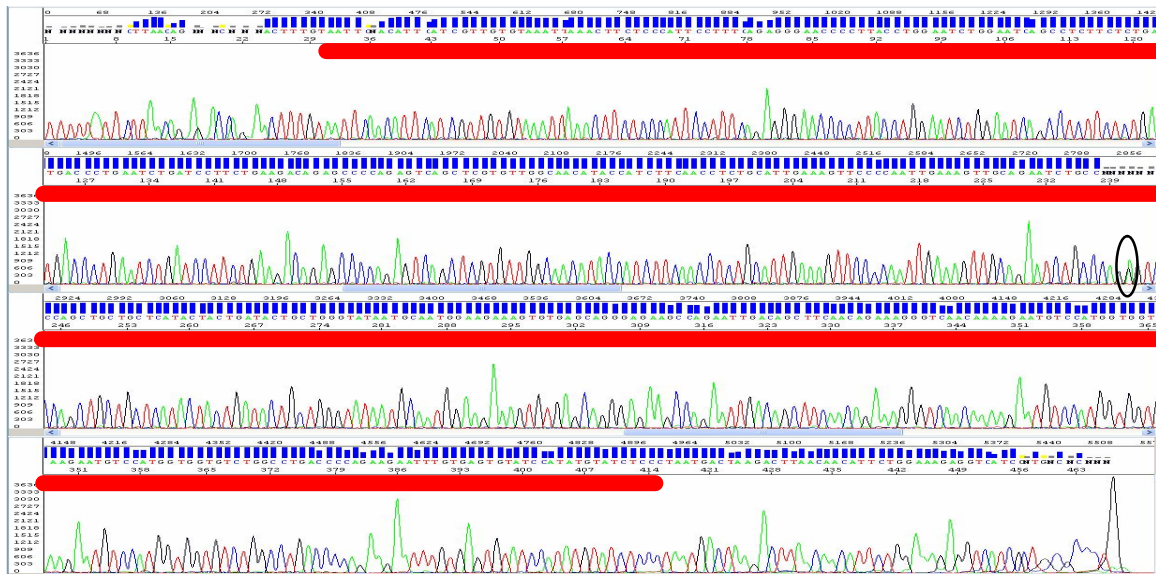


Figure 4: Sequencing of BRCA1 exon 16 in the forward orientation using US1 universal primer for the sequencing reaction. This amplicon is 486 bp long including US tails. Good quality sequence was obtained between base 44 and 475 inclusive. For comparison the analysable sequence for this amplicon is base 51 to 435 inclusive (indicated by red bar). A heterozygous sequence variation is present at base 261 (ringed).

9. TWO ROUND LABELLING

This system is most useful where labels are required on both strands of a double stranded PCR product (e.g. differentially labelled stands for use in fluorescent SSCP). A two primer PCR is run to produce a universally tagged product. This is then diluted and used as the template for a second round of PCR (much like nested PCR), but using an appropriately modified pair of US primers. This PCR is usually performed with a limited number of cycles (e.g. 15).

Because the required yield will vary from application to application it is inappropriate to give fixed conditions for this type of reaction; a certain amount of optimisation will almost certainly be required. The factors that can be varied are; the dilution factor applied to the primary PCR to produce an appropriate template concentration, the concentration of the US primers and the number of cycles used in the labelling reaction. However, in general it has been found most convenient to work with a fixed concentration of US primer and optimise the remaining parameters.

Example of two round labelling:

The following procedure was used to label ~500bp products with a VIC dye in one orientation. Two primer PCRs were set up as detailed in section 7 above. The PCR product was diluted 1:10000 in preparation for use as the template in a second round of PCR. The labelling reaction was setup as shown in tables 8 and 9:

Reagent	Final concentration		Quantity (μ l)
Template DNA (1:10000 dilution of primary PCR)	na		2
US1-VIC primer	30	fmol/ μ l	1
US2-unlabelled	30	fmol/ μ l	1
2x Amplitaq gold Mastermix	1	x	5
H ₂ O			1

Table 8: Setup for secondary PCR (labelling reaction).

Step	$^{\circ}$ C	Time		Cycle
Taq activation	95	10	min	
Amplification				} X15 cycles
Denature	95	5	sec	
Anneal	61	30	sec	
Extend	72	1	min	
Final extension	72	5	min	
Hold	15	∞		

Table 9: Thermal cycling conditions secondary PCR (labelling reaction).

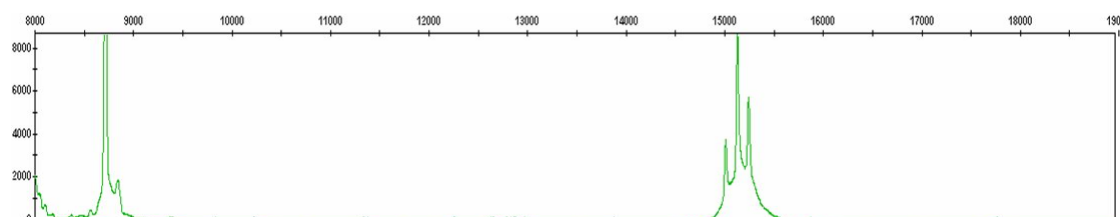


Figure 5: PCR product labelled using a two round labelling system. The products of the secondary (labelling) reaction were diluted 1:60 in water and run on an AB 3730 under partially denaturing conditions (conformation sensitive capillary electrophoresis). The three peak pattern indicates the presence of a heterozygous A>T sequence variation in this product.

10. THREE PRIMER SYSTEM

This system is most useful where a single label or tag is required to be added to a PCR product. In particular with fluorescent labelling for double stranded fragment analysis it enables any fragment to be labelled with any colour. This not only allows complete flexibility with respect to multiplexed or mixed analyses but can also substantially reduce the cost of fluorescent labelling.

10.1 Optimisation

As previously described the three primers used in this system are GS1, GS2 and a modified US1 primer (e.g. fluorescently labelled). The key to successful amplification using the three primer system is the relative concentration of the three primers in the PCR reaction (referred to as primer balance). The GS1 primer is used at a limiting concentration so that it is used up in the early rounds of PCR. This adds a US1 tail to the PCR product thus enabling the modified US1 primer take over (from GS1) in later rounds of the PCR.

Since it is the modified US1 primer that adds the required functionality to the PCR product (e.g. fluorescent label) the yield of labelled product can be controlled by varying the amount of US1 primer used in the reaction. As with any PCR there is a limit to product yield; thus, in practice, there is a limit to the amount of US1 primer that can be used. Above this limit adding more US1 primer will simply add to the pool of unused primer (i.e. primer flare in the case of fluorescent analysis), and increase the probability of primer dimers being formed. Similarly the appearance of dimers can often be controlled by varying the relative concentration of GS1 and GS2 primers.

We have found the most effective method for optimising the primer balance for these reactions is to use a standard amount of modified US1 primer and perform a cross titration of the concentrations of the GS1 and GS2 primers to determine the optimal yield of functional product. All primer sets optimised in this laboratory use 15fmol/ μ l of US1 primer and the initial optimisation uses 3, 9, 27, and 81fmol/ μ l of GS1 primer titrated against 40, 80, 160 and 320 fmol/ μ l of GS2 primer (figure 6). This scheme produces a satisfactory product yield for most primer sets. In some cases the concentration ranges for one or both of the GS primers need to be adjusted for a second round of optimisation to improve product yield or eliminate non specific products (e.g. primer dimer). Where this fails the primer pair is redesigned.

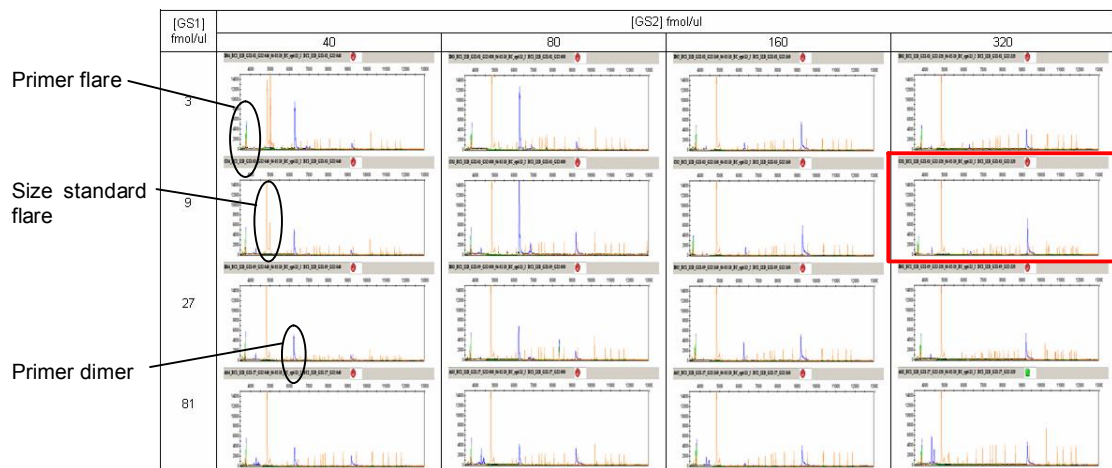


Figure 6: Cross titration of GS1 and GS2 primers for BRCA2 exon 23 using 15 fmol/ μ l of US1-FAM. A LIZ size standard has been used for electrophoresis and is shown in orange. The scales for all electropherograms are the same. In this case the amount of primer dimer present is inversely proportional to both GS1 and GS2 concentration. The best product yield and peak quality is given by a reaction containing 9 fmol/ μ l of GS1 and 320 fmol/ μ l of GS2 (indicated by red square).

10.2 GS primer mixes

For each amplicon a 5x working stock of mixed GS1 and GS2 primers is made according to the appropriate concentrations determined by the optimisation. For disseminated primer sets the information required to make these mixes is contained in the 'dilution schedule' worksheet of the supplied excel workbook. Each column of the table has a descriptive comment that can be viewed by mousing over the column header.

The mix is made in two stages; a primary and a secondary dilution coloured yellow and green respectively in the dilution schedule. The calculations are based on working from individual primer stocks all at 100 pmol/ μ l (or 100,000 fmol/ μ l). The primary dilution involves diluting the GS1 primer by a factor that is equivalent to the ratio of GS1:GS2 concentrations required in the final mix.

The secondary dilution involves mixing the two primers together and diluting down to a 5x working stock. The amount of stock to be made can be adjusted by altering the red 'Rxns' column ((c) in figure7) to the required number of reactions. The final quantity of mix will be enough for this number of 10 μ l reactions as well as 30 μ l waste volume. In general no other cells should be adjusted as all contain specific formulae. However, If original stocks are not at 100 pmol/ μ l the sheet can be amended accordingly by expanding columns indicated by (a) in figure 7.

Example dilution (refer to figure 7 below):

To make 100 reactions of 5x GS primer mix for amplicon BRCA1 exon 2 (BC1_02) the primary dilution should contain 2 μ l of BC1_02_fwd_US1_03 and 9.9 μ l of H₂O. The secondary dilution should contain 1.8 μ l of the primary dilution, 1.8 μ l of BC1_02_rvs_US2_05 and 226.3 μ l of H₂O, giving a final volume of 230 μ l – enough for 100 reactions (2 μ l each) + 30 μ l waste / dead volume.

25/01/2007				Rxn concentration (fmol/ μ l)		Primary dilution components (μ l)		Secondary dilution components (μ l)			Checks			Rxns
Fragment	fwd	rvs	GS1	GS1	GS2	GS1	H2O	Primary	GS2	H2O	[GS1]	[GS2]	1 ^o dilution vol	
BC2_02	01	01	Fwd	3	500	1	165.7	5.8	5.8	218.5	ok	ok	ok	100
BC2_03	01	01	Rvs	3	160	1	52.3	1.8	1.8	226.3	ok	ok	ok	100
BC2_04	01	01	Fwd	9	160	1	16.8	1.8	1.8	226.3	ok	ok	ok	100
BC2_05	01	01	Rvs	20	320	1	15.0	3.7	3.7	222.6	ok	ok	ok	100

Figure 7: Example of dilution schedule for GS primers. The date the spreadsheet was compiled is given in the top left hand corner (25/01/07). The components of the primary and secondary dilutions are shown in yellow and green respectively. If original stocks are not at 100 pmol/ μ l the sheet can be amended accordingly by expanding columns indicated by (a). Hidden columns (b) hold additional details about the dilution. Column (c) is used to define the amount of each mix that is required including 30 waste volume.

If robotic systems are in use the dilution schedule can be simply modified to give a .csv or worklist file that can be utilised for automated primer dilution.

10.3 PCR setup

PCRs are normally setup in 10 μ l volumes although this can be scaled if required. In practice it is simplest to make up a master mix containing the Amplitaq gold Mastermix and the appropriate US1 primer. 6 μ l of this mix should be used in a 10 μ l reaction meaning a PCR can be setup from just 3 components (DNA, GS primer and mastermix), greatly simplifying automation. A different mastermix can be made up for each required modification (e.g. FAM, VIC, NED etc)

Reagent	Final concentration		Quantity (μ l)
Template DNA (20 ng/ μ l)*	4	ng/ μ l	2
5x GS primer mix	1	x	2
2x Amplitaq gold Mastermix	1	x	5
US1 primer**	15	fmol/ μ l	1

Table 10: Standard 10 μ l PCR setup.

*In most cases 40ng will be suitable although varying the DNA concentration can help with problematic samples.

**Suitably labelled or tagged

Because all amplicons designed using this system are universally labelled, the risk of cross contamination is potentially increased. However careful use of normal precautions including spatial separation of pre and post PCR workflows with dedicated pipettes for each and the use of filter tips should be adequate protection in most cases.

10.4 Thermal Cycling

All the primer sets have been tested and optimised on a MJ research PTC-225 DNA Engine Tetrad. Standard thermal cycling conditions are given in table 11.

Step	$^{\circ}$ C	Time		Cycle
Taq activation	95	10	min	
Amplification				} X45 cycles
Denature	95	5	sec	
Anneal	61	30	sec	
Extend	72	1	min	
Final extension	72	5	min	
Hold	15	∞		

Table 11: Standard thermal cycling conditions for SPODS primers

It is possible that different PCR machines may give different results if the calibration varies significantly. Apart from temperature set-point variation, which should be resolved by regular calibration, there are three critical factors that may affect the PCR performance. Temperature set-point overshoot and time to equilibration will be largely machine specific but should remain reasonably constant for a particular instrument particularly with regular servicing. The primers have been designed with a high maximum annealing temperature (i.e. 68 $^{\circ}$ C) to minimise this issue. The ramping rate used on the MJ research PTC-225 DNA Engine Tetrad was set to the maximum (3 $^{\circ}$ C/sec). It is possible that a slower ramping rate may have a detrimental affect but a faster rate should not adversely affect the PCR performance.

Some PCR machines allow set-point temperatures to be determined by different methods. For example the MJ research PTC-225 DNA Engine Tetrad can use either block temperature or a formula to calculate the predicted temperature inside the tube or plate. SPODS primer pairs have been shown to work using either method during testing. However, if problems are experienced with PCR performance it is recommended that block temperature is used as a first step since this does not rely on calculations that may differ between machines.

11. UNTAGGED PRIMERS

Although this system has been specifically designed for use with tags and universal primers, there may be occasion to use untagged primers. For this reason the sequences of these primers have also been given along with other relevant information. For referencing purposes it is recommended that the system described in Section 4.1 is used with the omission of the tag reference to distinguish them from the tagged set.

PCRs should be setup as described in Sections 7.1 and 7.2.

It should be noted that the use of untagged primers will reduce the buffers designed into the amplicons. These buffers may be too short for robust analysis with some techniques. For such applications it is recommended that the tagged primer system is used.

12. OTHER POTENTIAL APPLICATIONS

Suitably modified US primers in conjunction with a **two** or **three primer system** could potentially be used to add functional tags to either or both ends of amplicons. This may be useful for PTT or MassCLEAVE analysis.

This system may also lend itself to a variety of multiplex applications although testing is, as yet, limited. For example a **four primer system** (using M13 tag instead of US tags) has been used to set up a semi quantitative assay to determine the relative copy number of MPZ and PMP22 sequences for the diagnostic evaluation of patients affected with HMS1 and HNPP (Poster 6.23 BSHG conference, JMG sept 2000 supp.1 ppS37). Here GS primers for a variety of different amplicons and both US primers were used in a single PCR reaction. All GS primers were used at limiting concentrations so that, except for the first few rounds of PCR, amplification of all the different products was primed with the universal sequences. Since this reaction is competitive with respect to the primers, the reaction is essentially semi-quantitative and theoretically more reliable than using a simple multiplex approach.

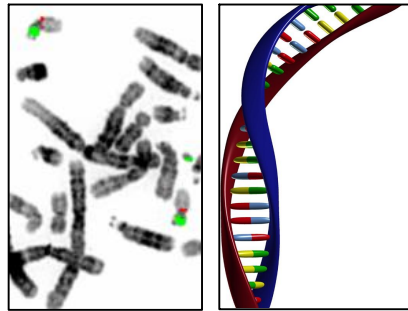
The potential of this system for bar-coding samples for analysis using massively parallel sequencing technologies is currently being investigated.

13. PRIMER SETS CURRENTLY AVAILABLE

The following primer sets are fully optimised. Full details including primer sequences and locations are available in Excel spreadsheet format. Please contact Chris.mattocks@salisbury.nhs.uk for further details.

BRCA1 – coding exons (34 amplicons)

BRCA2 – coding exons (45 amplicons)



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