CHAPTER 4.

COMPETITIVE PCR.

PART 1. INTRODUCTION.

Summary.

This chapter describes the development of competitive PCR techniques for the estimation of template copy numbers, and the practical application of these techniques to the field of rumen bacterial ecology and biotechnology.

Experiments were also carried out to determine whether, in fact, non-competitive PCR could be used for quantitation.

Competitive PCR.

PCR, because it is an exponential reaction, is exceedingly sensitive to very small differences in reaction conditions, both to differences in the actual reaction mixture, and to temperature differences during the reaction. For this reason it had been considered by many researchers (Chelly *et al*, 1990; Gilliland, 1990; Kellogg *et al*, 1990; Wang and Mark, 1990.) that it was not possible to accurately determine the template copy number from measurement of the amount of amplification product alone. For this purpose, the technique of competitive PCR developed by Gilliland *et al*, (1990) was considered to provide more accurate and reliable results than the conventional PCR reaction. The methods used by these researchers are described in Chapter 1 (page 9). However, in summary, competitive PCR is performed by simultaneously amplifying two DNA fragments in a single reaction. The ratio of the two amplification products is then determined, and this can be related to the ratio of the original template DNA fragments. This ratio is independent of small differences in reaction conditions, as it is determined

solely by the relative amplification efficiencies of the two reactions, and these should be equally affected by any differences in the reaction conditions.

The published methods describe two main types of control sequence. The first has the same primers as the target sequence, but differs from the target sequence either in length, having a number of base pairs deleted from the sequence (e.g. Chelly, 1988), or in the possession or absence of a unique restriction site (e.g. Gilliland, 1990). The second is an entirely different sequence, with different primers (e.g. Kellogg, 1990). The methods of Chelly and Kellogg are not strictly competitive PCR, as, although control sequences are co-amplified with target sequences, the product ratios are not computed, and quantitation is only valid if the PCR amplifications are kept within the exponential stage. For this reason, the methods of Gilliland *et al* were considered to be preferable, particularly because, when amplifying a large number of samples with differing levels of target DNA, it is difficult to ensure that no samples will amplify beyond the exponential stage.

The use of a control sequence that could be differentiated from the target by restriction digest of the amplification products was rejected as introducing an unnecessary extra step into the proceedings. However, the use of a control sequence possessing the same primers as the target sequence seemed to offer advantages in simplicity, cost saving, and, since the sequences had considerable identity, likelihood of similar amplification efficiencies. Therefore the initial experiments were designed to produce and test a deletant of the target sequence as a control.

PART 2. EXPERIMENTAL WORK.

4.2.1. TWO PRIMER COMPETITIVE PCR.

This section deals with competitive PCR where the control DNA sequence used was a deleted version of the target sequence. The deletion was made in the centre of the control sequence so that the target and control had common sequence at each end, and the same two primers could be used to amplify both sequences.

4.2.1(A). TARGET AND CONTROL SEQUENCES FOR *P. RUMINICOLA* AR20.

Materials and Methods.

The target sequence chosen for competitive PCR of *P. ruminicola* AR20 was the same sequence of the plasmid pJW4 as had been used for tracking experiments. (See Chapter 3 Section.2.1). The control sequence was a deletion of pJW4 constructed by linearising the plasmid at a unique *Stu* I site within the target sequence (see Appendix 1), enzymically deleting nucleotides from the free ends, and re-circularising the deleted fragment.

(i). Linearisation of pJW4 with *Stu* I.

1 µg pJW4 DNA was incubated with 10 units *Stu* I in 400 µl of the manufacturer's buffer solution for 2 hr at 37°C. Linearisation was confirmed by electrophoresis of a 10 µl aliquot on 1% agarose gel.

(ii). Deletion of linearised pJW4.

10 µl aliquots of the above reaction containing approx. 25 ng DNA were diluted to 50 µl in REB. The mixtures were incubated at 37°C for 60, 90 and 120 min with 0.5 units *E. coli* DNA Polymerase I, and then heated to 70°C for 5 min. to de-activate the

enzyme. The reactions were then incubated with 0.5 units of the Klenow fragment of *E. coli* DNA Polymerase I at 37°C for 5 min in the presence of 50 mM dNTPs, to fill in single stranded overhangs and produce flush ends to the deleted sequence. The DNA was then precipitated with 0.1 vol. 3M sodium acetate (pH 4.5) and 2.5 vol. ethanol.

(iii). Re-circularisation of deleted fragments.

The precipitated DNA was redissolved in 50 µl ligation buffer, with 0.5 units T4 ligase and incubated overnight at room temperature.

(iv). Transformation of deleted plasmids.

Transformation was by electroporation of *E. coli* K803 with 1 µl of the above ligation. After incubation at 37°C for 1 hr, cells were centrifuged, resuspended in approx. 0.1 ml SOB medium, and spread on LB + ampicillin plates to select transformants.

(v). Restriction enzyme digests of deleted plasmids.

Plasmid DNA was extracted from cultures of three 60-min deletion colonies, and three-90 min deletion colonies by the alkaline lysis method. Aliquots of DNA from each deletant and from pJW4 were digested with 2 units each of *Acc* I and *Pvu* II. Digests were electrophoresed on agarose gel to determine relative sizes of DNA fragments.

Results.

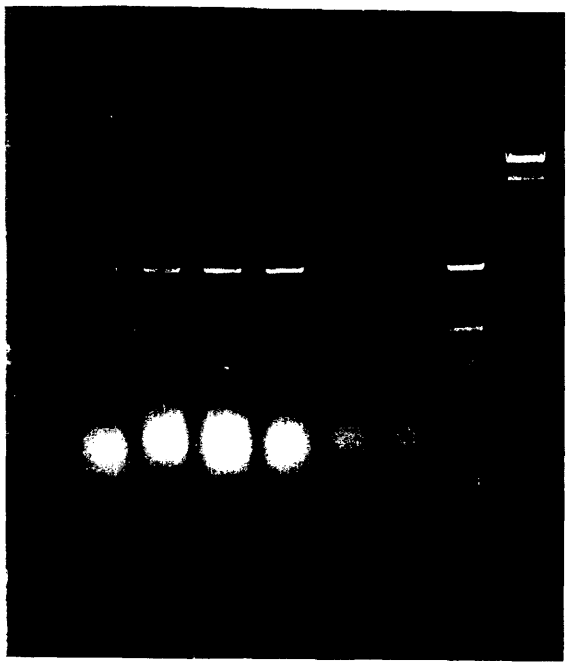
(i). Transformation of deleted plasmids.

13 colonies were obtained from the 60-min deletion, 4 from the 90-min deletion, and none from the 120 min deletion.

(ii). Restriction enzyme digests of deleted plasmids.

Fig. 4.2.1

Restriction enzyme digests of deleted plasmids.



Lanes 1-3 show Pvu II/ *Acc* I digests of 60-min deletant plasmids.
Lanes 4-6 show *Pvu* II / *Acc* I digests of 90-min deletant plasmids.
Lane 7 shows Pvu II/ *Acc* I digest of pJW4.
Lane 8 shows λ phage DNA cut with *Hind* III as a size marker.

Results of the restriction digest of the deletant plasmids are shown in Figure 4.2.1. The middle band of DNA contained the fragment that had been reduced in size by the deletion - religation process. The plasmid in lane 5 showed the greatest deletion, approximately 100 bases judged by visual inspection of the gel. This was selected as the control plasmid for *P. ruminicola* AR20, and named pJD6. The largest deletion was chosen to most easily distinguish the control and target PCR fragments

4.2.1(B). COMPETITIVE PCR OF *P. RUMINICOLA* AR20 DNA.

Materials and Methods.

DNA from the control and target plasmids, pJW4 and pJD6, was co-amplified in the same reaction using the primers MTP1A and PVP2ex, which had annealing sites on both plasmids. Both target (pJW4) and control (pJD6) plasmids were also amplified separately using the same primers. Amplification was for 20 cycles using cycle times and temperatures as specified for pJW4 DNA in Table 3.2.1.

Template DNA amounts are shown in Table 4.2.1. Reactions were performed in duplicate..

Table 4.2.1.

Competitive PCR templates.

Tube Number	pJW4 DNA ng	pJD6 DNA ng
1	1.0	-
2	0.1	-
3	0.01	-
4	-	1.0
5	-	0.1
6	-	0.01
7	1.0	1.0
8	0.1	0.1
9	0.01	0.01

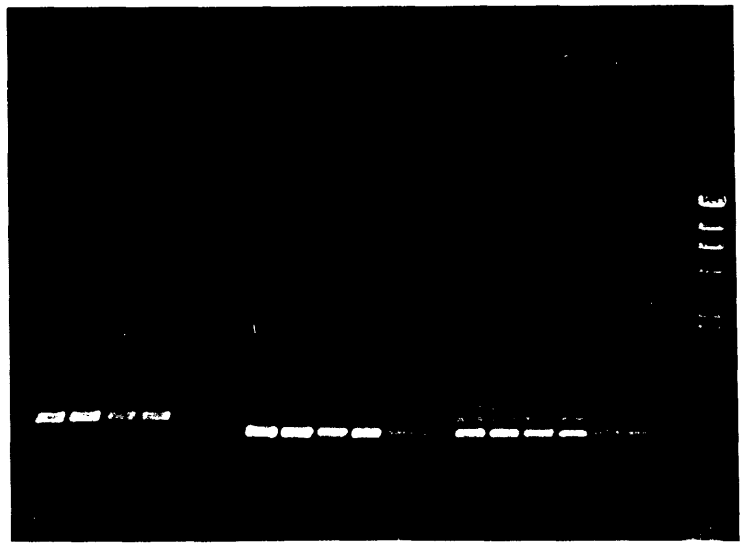
After amplification, 7 µl of each reaction was electrophoresed on 1% agarose gel. The gel was stained with ethidium bromide, photographed and the negative scanned by laser densitometry to determine relative concentrations of amplification products.

Results.

The results of the PCR amplification are shown in Fig.4.2.2. Except at low levels of amplification products, a third DNA band, longer than the specific amplification product bands, appeared as an amplification product when target and control were co-amplified.

The results of the scan of the negative of Figure 4.2.2 are shown in Tables 4.2.2. and 4.2.3, and mean scan results are shown in Graphs 4.2.1 and 4.2.2.. These show that the formation of the third DNA product was accompanied by a decrease in the rate of formation of the specific amplification products. In fact production of the specific amplification product of pJW4, which was present at a lower concentration than that of pJD6, ceased with the appearance of the third band.

Figure 4.2.2



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Lanes 1-6 show duplicate amplifications of 1.0, 0.1, 0.01 ng pJW4 DNA.
Lanes 7-12 show duplicate amplifications of 1.0, 0.1, 0.01 ng pJD6 DNA.
Lanes 13-18 show duplicates of 1.0, 0.1, 0.01 ng pJW4 DNA + 1.0, 0.1, 0.01 ng pJD6 DNA.
Lane 20 shows λ phage DNA cut with *Hind III* as a size marker.

Table 4.2.2.

Scan Results of Separate Amplifications of pJW4 and pJD6.

	Scan Results, Au mm	
Template DNA ng	pJD6	pJW4
0.01	0.512	0.362
0.01	0.569	0.363
0.1	1.134	0.703
0.1	1.021	0.661
1.0	1.184	1.109
1.0	1.179	1.074

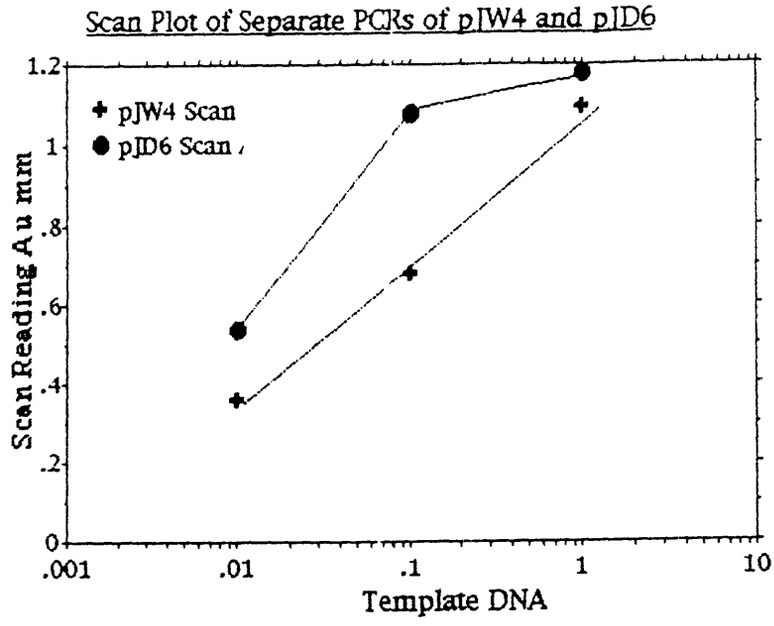
Table 4.2.3.

Scan Results of Co-Amplifications of pJW4 and pJD6

	Scan Results, Au mm		
Template DNA ng	**pJD6**	**pJW4**	**Unknown**
0.01	0.541	0.397	0.04
0.01	0.548	0.329	0.04
0.1	0.664	0.347	0.260
0.1	0.662	0.401	0.296
1.0	0.748	0.356	0.362
1.0	0.721	0.361	0.380

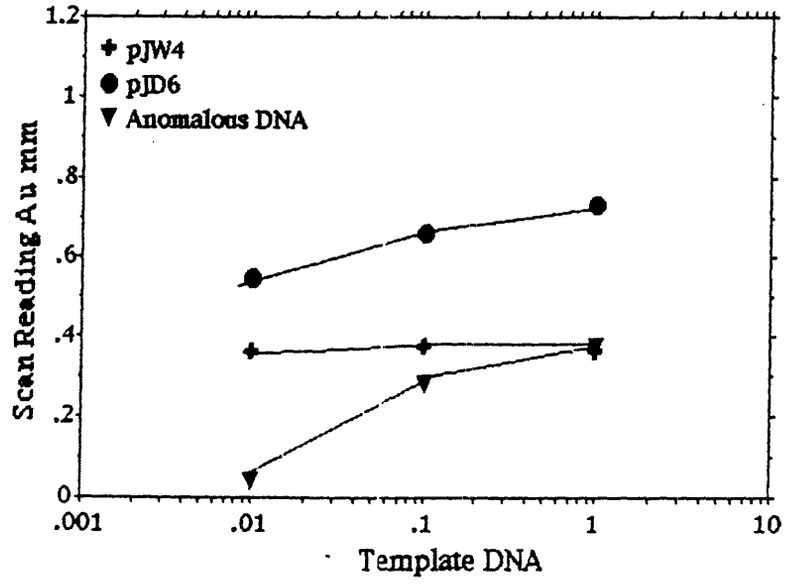
Graph 4.2.1.

Scan Plot of Separate PCRs of pJW4 and pJD6



Graph 4.2.2.

Scan Plot of Competitive PCRs of pJW4 and pJD6

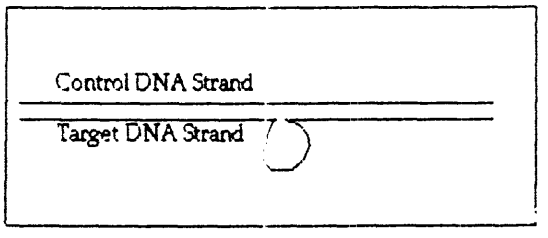


4.2.1.3. INVESTIGATION OF UNKNOWN CO-AMPLIFICATION BAND.

It seemed probable that the third band of DNA that appeared when pJW4 and pJD6 were co-amplified was a heteroduplex of the two specific amplification products, which electrophoresed more slowly than the specific amplification products because of its shape. (See Figure 4.2.3.).

Figure 4.2.3.

Conformation of Heteroduplex DNA.



The heteroduplex product forms when the complementary single strand sequences of target and control DNA strands anneal. Because the target strand is longer than the control strand, the unpaired bases form a loop of single stranded DNA bulging out from the paired strands.

Materials and Methods.

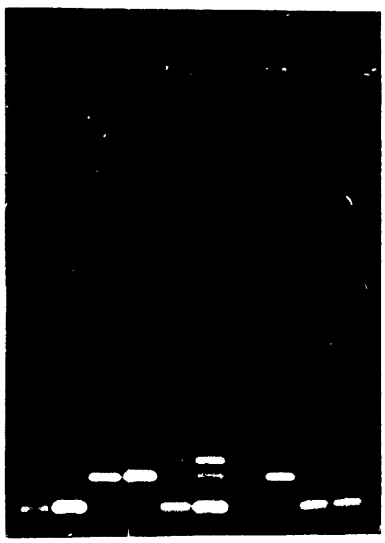
To test this hypothesis, pJW4 and pJD6 were co-amplified for 20 cycles, using 0.05 ng of each plasmid DNA, and primers GA3I and PVP2ex (see Appendix 1). 5 µl of the amplification products was electrophoresed on 4% acrylamide gel to separate the three DNA bands, and the gel was stained with ethidium bromide to visualise the bands. These were then excised separately from the gel and soaked in an equal volume of distilled water overnight to extract the DNA. 1 µl samples of the eluate from the two shorter bands, and 5 µl samples from the longest band were then re-amplified by PCR for 25 and 30 cycles. 0.005 ng of pJW4 and pJD6 DNA were amplified separately under the same conditions for comparison

Results.

Results are shown in Figure 4.2.4. It can be seen that the two faster DNA fragments produced bands identical to specific bands produced from pJD6 and PJW4 respectively, while the slowest fragment reproduced the three bands, pJD6 fragment, pJW4 fragment and presumed heteroduplex.

Fig. 4.2.4.

PCR Amplifications of Co-Amplification DNA Products



1 2 3 4 5 6 7 8 9 10

Lanes 1 & 2 show 25 and 30 cycle re-amplifications of shortest amplification product
Lanes 3 & 4 show 25 and 30 cycle re-amplifications of middle amplification product.
Lanes 5 & 6 show 25 and 30 re-amplifications of longest amplification product
Lanes 7 &-8 show specific amplification products of pJW4.
Lanes 9&10show specific amplification products of pJD6.

These results showed that the unknown band comprised specific amplification products from both pJW4 and pJD6, running as a single band. This confirmed that the third DNA band consisted of a heteroduplex of the two specific amplification products.

Discussion.

The appearance of a heteroduplex band in the co-amplification of target and control sequences DNA, could be avoided by keeping amplification at a low level. However, it was felt that a system which could avoid the problem of heteroduplex formation would be preferable. There were two main reasons: first, errors of measurement would be increased by 50% if three DNA bands rather than two were scanned. Second, when PCRing complex mixtures of DNA such as rumen contents, non-specific amplification products were commonly observed (see Figure 3.2.7. for example). These were normally ignored, as they did not arise from the organism being tracked. However, a system producing a possible heteroduplex DNA band as well as possible non-specific bands was felt to be too complicated and susceptible to errors of interpretation.

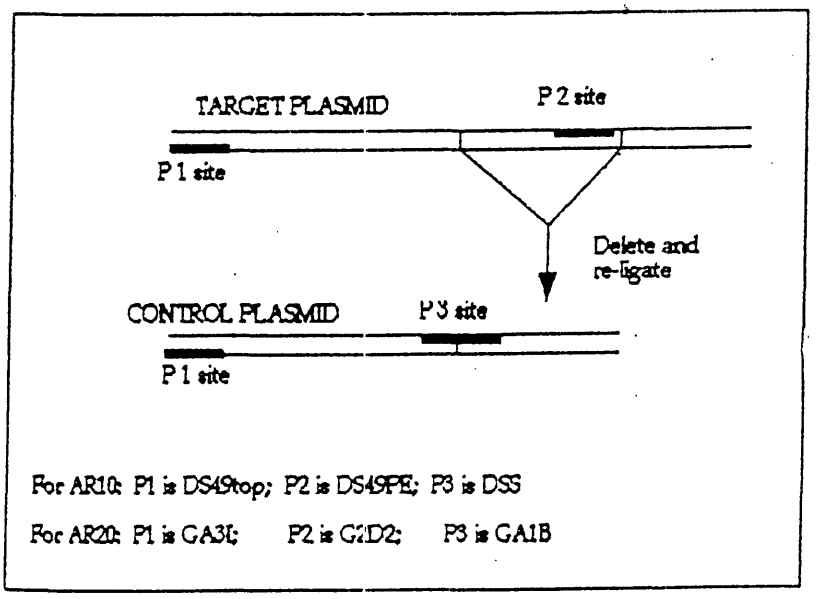
4.2.2. THREE PRIMER COMPETITIVE PCR.

Since the two-primer competitive PCR system had proved unsatisfactory, a different system was required. It was hypothesised that if the same target and deletant control plasmids were used, the problem of heteroduplex formation might be overcome by changing the common reverse primer to primers which were specific, one for the target sequence, and one for the control sequence.

Accordingly a system was tested using three primers (see Fig 4.2.5), which had primer annealing sequences on the AR20 target and control plasmids already produced for the two primer system.

Fig. 4.2.5.

Primer Annealing Sites for Three Primer Competitive PCR.



Part of the target plasmid DNA is shown with both primer sites, and the section of DNA which is deleted to form the control sequence, and which contains the site for the P2 target primer.
Part of the control plasmid DNA is shown with the religation point, and the P3 primer site spanning the religation point.

The P1 primer annealed to a common sequence present on both target and control plasmids.

The P2 primer was specific for the target plasmid, and annealed to the distal end of the sequence that had been deleted from the control plasmid.

The P3 primer was specific for the control plasmid at conditions of high annealing stringency, and annealed across the deletion -religation point on the control plasmid.

4.2.2(A). PRIMERS AND CONTROL SEQUENCES.

(i). Primers for *P. ruminicola* AR20.

The pJD6 plasmid, containing the control sequence for the competitive PCR of AR20, was sequenced by the cycle sequencing method between the two primers MTP1A and PVP2ex, using MTP1A as the hot primer, to find the site and extent of its deletion. It was found that 139 bases had been deleted (see Appendix 1). Primers were selected as shown in Fig. 4.2.5.

Primer GA3I was the common primer.

Primer G2D2, specific for the target sequence, was selected within that section of the target plasmid that had been deleted to form the control plasmid.

Primer GA1B, specific for the control sequence, was selected to span the deletion-religation site on the control plasmid.

Primers G2D2 and GA1B were selected to have approximately equal free energies of association.

See Appendix 1 for primer sites and sequences.

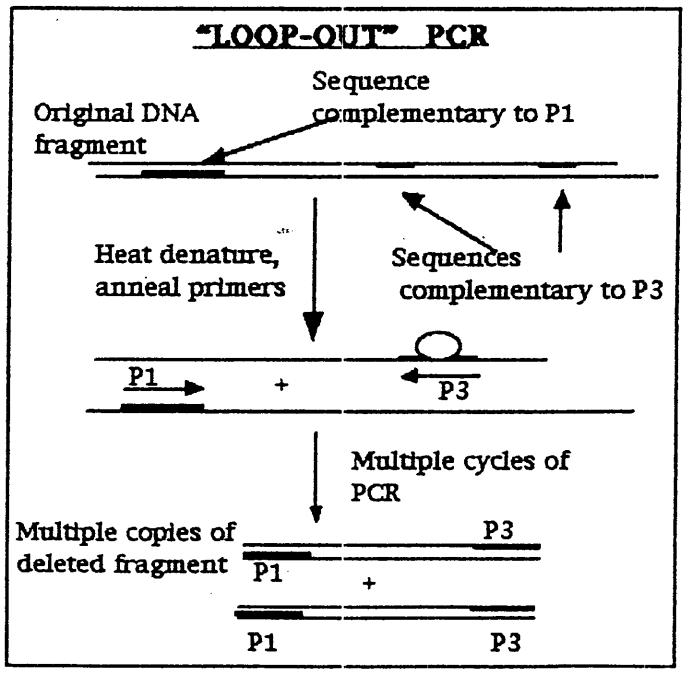
(ii). Target and Control Sequences for B. fibrisolvens AR10.

The target sequence for *B. fibrisolvens* AR10 was the DS49PE-DS49top fragment of pDS49 that had been used for tracking (see 3.2.2)

The control sequence for *P. ruminicola* AR20 had been prepared by cutting the target DNA at a unique restriction site on the plasmid containing the target sequence. The target sequence that had been used for AR10 contained no such unique site. Therefore a different method had to be used to obtain a deletion.

The method developed was the reverse of the insertional mutagenesis method of Kamman *et al*, (1989). That method used as a PCR primer a single stranded piece of DNA that annealed to its target at both ends, but contained non-complementary sequence between. This non-complementary sequence was copied into the amplified product. The "loop out" deletion method developed here used a primer which was complementary to two short sequences of DNA on either side of the desired deletion (see Fig 4.2.6). As only half the primer anneals to each complementary site on the template, PCR amplification using this primer was performed at low annealing stringency. Amplification gave a deleted product that could be cloned into a plasmid for use as a competitive PCR control sequence. The "loop out" primer then became the reverse primer for competitive PCR at high annealing stringency.

Figure 4.2.6.

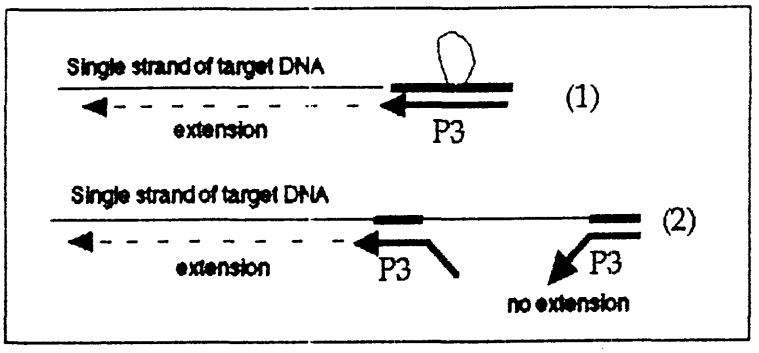


The P3 primer is shown annealing to the two separated halves of its complementary sequence, leaving a loop of non-complementary DNA. Polymerisation takes place in the direction of the arrows resulting in the shortened fragments of DNA shown at the bottom of the diagram.

It was later realised that the "loop out" mechanism was not necessarily the way the deletants were produced, (see fig. 4.2 7.). It was equally possible that only the forward half of the "loop out" primer annealed to the template. The amplification products would then comprise the full deletant. Both possible mechanisms would give the same result, and it is possible that both operated to some extent.

Fig. 4.2.7.

Possible Annealing Mechanisms of P3 Primer to Target Sequence.



(1) shows the primer annealing to the two separated halves of its complementary sequence.
(2) shows two primers each annealing to one half of the separated complementary sequence. If the 5' end of the primer anneals, extension will occur, producing the same shortened fragment produced by the first annealing mechanism

(iii). Copy Numbers of Target Sequences on Genome.

Before meaningful results can be obtained from competitive PCR, the copy number of the target sequence on the genome of the organism under investigation must be known. The pJW4 sequence had been shown by Jane Woods (1989) to be present only once on the *P. ruminicola* AR20 genome. The copy number of the DS49 sequence on the *B. fibrisolvens* genome was determined by standard hybridisation techniques.

Materials and Methods.

(i). Preparation of Deletant Fragments by PCR.

Sequence for primers DSS and DSG was selected from the pDS49 sequence. Both had 12 bases complementary to pDS49, downstream of, and immediately adjacent to DS49PE, and 12 bases complementary to pDS49 approximately 100 bases upstream of DS49PE (see Appendix 1).

Approximately 1 ng of pDS49 plasmid DNA was used as template. The annealing temperature was 35°C. 20 cycles of PCR were performed.

Similar deletants of pGAR 11 and pGOB4 were produced as control sequences for AR29 and OB156.

(ii). Cloning of Amplification Products.

Amplification products were electrophoresed on 1% agarose to determine their concentrations. They were then ligated into *Sma* I cut pUC18 using the Pharmacia Sure-Clone kit according to the manufacturer's instruction. Approx. 1 ng of the ligation product was electroporated into electro-competent *E. coli* K803. Electroporated bacteria were spread on to LB + ampicillin agar plates to select recombinants.

DNA from resultant colonies was amplified by PCR using primers DS49top and DSS to test for the presence of the control fragment.

Attempts were also made to clone the amplified DS49 fragment into *E. coli*, to produce target and control plasmids of comparable length.

(iii). Copy Numbers of Target Sequences on Genome.

To determine the copy number of the pDS49 sequence on the AR10 genome, AR10 genomic DNA was digested with the restriction enzymes *Rsa* I and *Hind* III, neither of which have recognition sequences within the DS49top -DS49PE sequence, and the

resultant fragments were separated on a 1% agarose gel. DNA was transferred from the gel to a nylon filter by Southern blotting, and $\gamma^{32}P$ labeled DS49top - DS49PE amplification product was hybridised to the genomic digests.

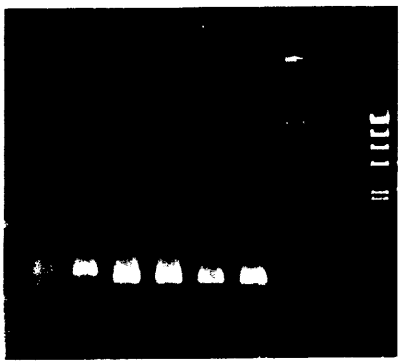
Results.

(i). PCR Production of Deletant Fragments.

Figure. 4.2.8. shows amplification products of the target plasmid DNA at low annealing stringency, using DSG and DSS primers complementary to two separated 12 base sites on the target sequence. Amplification products migrated with the predicted mobility for their theoretical sizes.

Figure 4.2.8.

Amplification Products of pDS49 from Different Primer Pairs.



Lane 1 shows the amplification product from DS49top and DS49PE.
Lane 2 shows the product from DS49top and DSG
Lane 3 shows the product of DS49top and DSS
Lane 4 Shows λ phage/*Hind* III size marker.

(ii). Cloning of Amplification Products.

7 colonies were obtained from cloning the DS49top-DSS plasmid, of which 6 proved to be identical and to contain the correct fragment. The plasmid was named pDSS. No colonies grew from cloning the DS49top-DSG plasmid.

Attempts were made to ligate the DS49top-DS49PE PCR target fragment into pUC 18 to produce target and control plasmids of comparable size. However, although the ligation was successful, this construct could not be cloned, in spite of many attempts in several different strains of *E. coli*. Similarly, it proved impossible to clone the pJW4 and pJD6 PCR fragments and the deletants of pGAR11 and pGOB4 into pUC 18.

(iii). Copy Number of AR10 Target Sequence on AR10 Genome.

The results of the Southern blot hybridisation can be seen in Fig. 4.2.9.

Figure 4.2.9.

Southern Blot Hybridisation of AR10 Genomic DNA

Lane 1 shows a *Hind* III digest of AR10 genomic DNA.
Lane 2 shows an *Rsa* I digest of AR10 genomic DNA.
Lane 3 shows the P^{32} labeled DS49top-DS49PE probe
Lane 4 shows λ phage/*Hind* III size marker
Lane 5 shows the autoradiograph of the probe hybridised to the *Hind* III digest
Lane 6 shows the autoradiograph of the probe hybridised to the *Rsa* I digest

Both autoradiographs show a single hybridisation position on the genomic DNA, denoting that the DS49 sequence appears only once on the AR10 genome. Consequently the copy number of target sequences in a carefully measured quantity of genomic or whole cell DNA would be equal to the number of copies of the genome.

The AR10 genomic insert in the pDS49 plasmid is a fragment from a *Hind* III digest of AR10 inserted into the *Hind* III site of the pUC multiple cloning region. The primers are taken from near the ends of the insert. Therefore, as shown, the probe and the genomic fragment to which it hybridised are nearly the same length.

The target sequences for *B.fibrisolvens* OB156 and *P. ruminicola* AR29 were similarly shown to be present only once on their respective genomes (data not shown.

4.2.2(B). TESTING THE THREE PRIMER SYSTEM.

(i) Comparison of Two Primer and Three Primer Competitive Systems.

It had already been shown that the two primer competitive system produced specific amplification products from both target and control templates when these were co-amplified. It was necessary to test the three primer system to see whether it provided satisfactory co-amplification, and whether or not heteroduplex products appeared.

Materials and Methods.

Co-amplification of target and control DNA with three primers, GA3I, GA1B & G2D2 was performed using 13, 15, 17, 19, 21, 23 & 25 cycles.

The common primer, GA3I, was added to a concentration of 30 mM, while the other two primers were each at 20 mM.

Template was 0.5 ng pJW4 DNA + 0.5 ng pJD6 DNA.

The same co-amplifications were performed using primers MTP1A and PVP2ex for comparison.

Cycle times and annealing temperatures were as for primers G3I and G2D2 in Table 3.2.1.

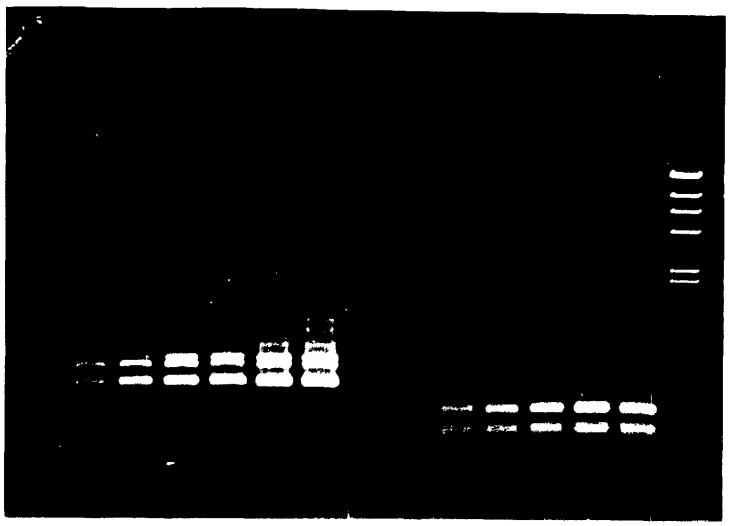
Results.

Results of the electrophoresis of the amplification products of all reactions can be seen in Figure 4.2.10.

It can be seen that after 17 cycles, the heteroduplex band was clearly visible when the two primers, MTP1A & PVP2ex, were used. With the three primer system, although there was accumulation of non-specific amplification products after higher amplification, there was no trace of a specific heteroduplex band.

Figure 4.2.10.

Co-Amplification of pJW4 and pJD6 with Different Primer Sets.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Lanes 1-7 show amplification products from 13, 15, 17, 19, 21,23 & 25 cycles of PCR using primers MTP1A & PVP2ex.
Lanes 8-14 show amplification products from 13, 15, 17, 19, 21, 23 & 25 cycles of PCR using primers G3I, GA1B & G2D2.

(ii). Investigation of Possible Heteroduplex DNA in the Three Primer System.

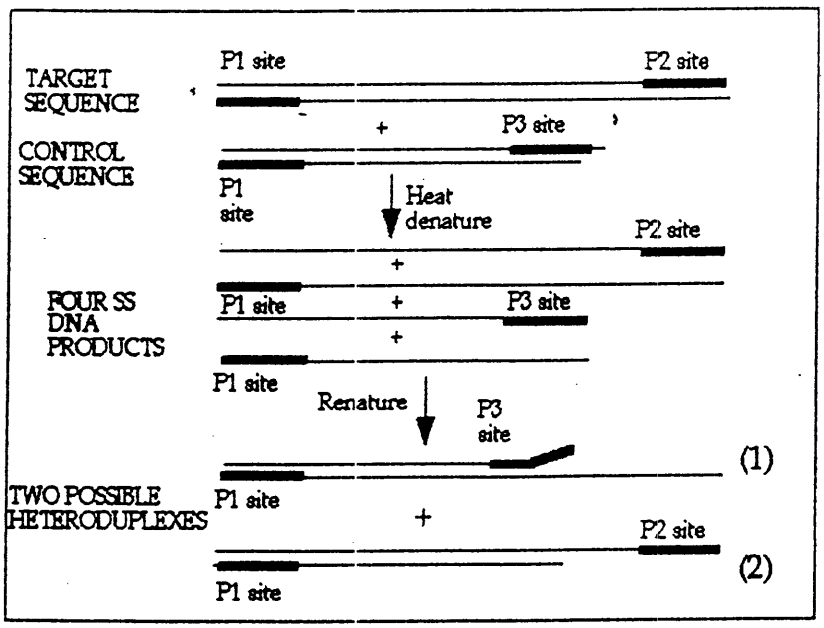
Although the three primer competitive PCR system showed no anomalous third band, there was some concern that heteroduplex products might be formed which migrated on the gel at the same mobility as the longer specific amplification product.

Heteroduplex DNA is most logically formed at the primer annealing stage of the PCR reaction. The primer is present in the reaction mix at a much higher molar concentration than the template DNA, and easily outcompetes the template and amplification products for annealing sites at early stages of the reaction. However, as

amplification continues and the concentration of amplification products increases, the tendency for the amplification products to anneal to each other, rather than the primer, increases. When only one template is being amplified, this merely results in a slowing down of the amplification rate. However, when two products which are the same for a large part of their length are being amplified, heteroduplexes will form. With the two heterologous DNA strands, from the two primer system, which were the same at both ends, and only differed in the middle, the formation of the heteroduplex blocked the primer sites, and prevented further amplification

Fig. 4.2.11.

Possible Heteroduplexes from Three Primer PCR.



The two possible heteroduplex molecules from the single stranded products of heat denatured target and control DNA are shown. Heteroduplex (1) has the P3 control primer site partially annealed to the target sequence, whereas the P2 primer site is free in heteroduplex (2).

The heterologous strands in the three primer system were identical at one end only, (see Fig. 4.2.11.). Of the two possible heteroduplex products, (2) was of no concern, as Taq polymerase is known to have template clearing action, (Innes,1989), and the P2 primer site is completely free. However, with (1), only half the P3 primer site is free, and it was considered possible that there could be a blockage of synthesis from this site, and heteroduplexes might accumulate.

Materials and Methods.

To test for the presence of heteroduplex (1) in the amplification products, pJW4 and pJD6 DNA were co-amplified for 15 cycles using primers GA1B, G2D2 and G3I, with GA1B labeled with γ^{32}P ATP. The amplification products were electrophoresed on agarose gel stained with ethidium bromide, to visualise all products; and on acrylamide gel with subsequent autoradiography, to visualise any product incorporating the P3 primer.

Templates: 0, 0.2, 0.4, 0.6, 0.8, and 1.0 ng of both pJW4 and PJD6

Results.

Figure 4.2.12. shows the results from both agarose and acrylamide gels. It can be seen that the agarose gel shows the two predicted specific amplification products of pJW4 and pJD6, whereas the autoradiograph of the acrylamide gel displays only one band, showing that all the amplification product incorporating the primer GA1B traveled as a single band under electrophoresis.

If any heteroduplex product had been formed, it would have been longer than the specific amplification product of pJD6, and would be expected to migrate more slowly

under electrophoresis. However, there was concern that such a heteroduplex might migrate at the same rate under electrophoresis as the pJW4 specific amplification product. The appearance of a single labeled DNA band on the acrylamide gel, corresponding to the shorter pJD6 product, demonstrated that there had been no formation of such heteroduplex.

Figure 4.2.12.

Amplification Products of Three Primer PCR of pJW4 and pJD6.

(i). Agarose Gel Stained with Ethidium Bromide.

1 2 3

Lanes 1and 2 show co-amplification products of pJW4 and pJD6 from primers GA1B, G2D2 and GA3I. GA1B was labeled with ^{32}P
Two amplification products are present.
Lane 3 Shows λ phage/*Hind* III marker

(ii). Autoradiograph.

1 2

Lanes 1 and 2 show co-amplification products of pJW4 and pJD6 from primers GA1B, G2D2 and GA3I. GA1B was labeled with ^{32}P
A single labeled product is present.

(iii). Co-Amplification Product Ratios.

It had been determined that co-amplification of target and control plasmids produced amplification products from both target and control templates. However, it was necessary to confirm that the amounts of these products bore some relationship to the amount of template DNA, and that the ratio of amplification products bore a definite relationship to the ratio of the two DNA templates.

Materials and Methods.

Varying concentrations of pDS49 and pDSS plasmid DNAs (see Table 4.2.4) were co-amplified for 18 cycles of PCR with the common primer, DS49top, labeled with γ^{32}P ATP. At higher ratios of pDS49 to pDSS, the concentration of pDSS DNA was reduced so as to keep the total template DNA within a narrow range of concentrations.

6.0 µl ficoll loading buffer was added to each tube, and 4.0 µl aliquots of each amplification mix were loaded onto 1 mm thick 4% acrylamide gel for electrophoresis. After electrophoresis the gel was dried and autoradiographed, and the autoradiograph scanned by laser densitometry at three positions across each DNA band. Ratios of amplification product concentrations were determined, and plotted against ratios of template DNA concentrations.

The co-amplification was performed on DNA extracted from plasmids by the alkaline lysis method. Plasmid DNA extracted by this method is not of a very high purity, so template concentrations are only approximate.

Table 4.2.4.

Plasmid DNA concentrations and Ratios for Competitive PCR.

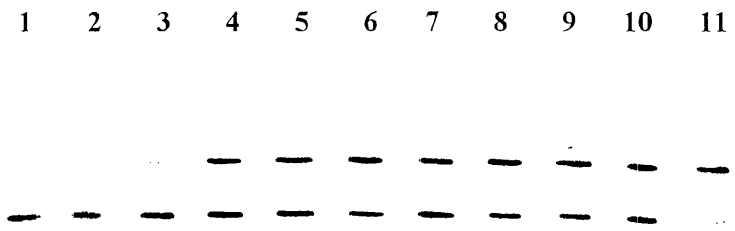
Tube no.	pDSS DNA ng	pJW4 DNA ng	Template Ratio
1	0.04	0.01	4:1
2	0.04	0.02	4:2
3	0.04	0.03	4:3
4	0.04	04	4:4
5	0.04	0.05	4:5
6	0.04	0.06	4:6
7	0.04	0.07	4:7
8	0.04	0.08	4:8
9	0.02	0.045	4:9
10	0.02	0.05	4:10
11	0.02	0.055	4:11
12	0.02	0.06	4:12

Results.

A photograph of the autoradiograph of the co-amplification products of pDS49 and pDSS at varying ratios of target (pDS49) and control (pDSS) templates is shown in Fig. 4.2.13. A plot of template ratio against product ratio determined from densitometer readings is shown in Graph 4.2.3. Full densitometer readings are shown in Appendix 3.

Figure 4.2.13.

Autoradiograph of Co-Amplifications of pDS49 and pDSS.

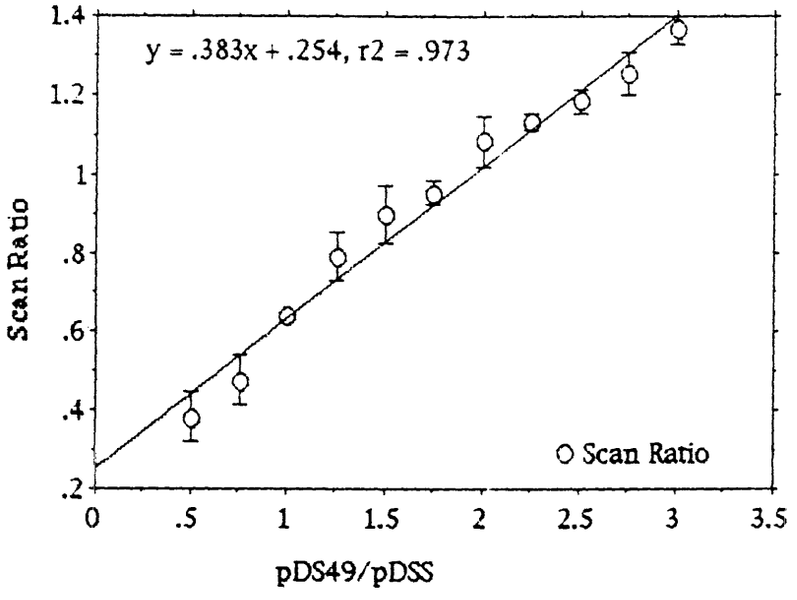


Lanes 1-8 show pDSS template concentration of 0.04 ng / 20 µl reaction,
and pDS49 at 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07 & 0.08 ng / 20 µl reaction.
Lanes 9-12 show pDSS template concentration of 0.02 ng / 20 µl reaction,
and pDS49 at 0.045, 0.05, 0.055 & 0.06 ng / 20 µl reaction.

The autoradiograph shows that as the template ratio of pDS49 DNA relative to pDSS DNA increased the density of the band on the autoradiograph representing the specific amplification product of pDS49 increased. Concomitantly, the density of the pDSS specific product band decreased. There was a tendency for the total amount of DNA produced by the amplification to rise with the amount of template DNA, although there were obvious variations in amplification efficiencies between reactions.

Graph 4.2.3.

Template Ratios vs Scan Ratios for pDS49 & pDSS.



Ratios of the areas of each peak, measured by laser densitometry from the autoradiograph of amplification products, are plotted against the ratios of pDS49/pDSS in the PCR template. The line of best fit and the slope of the line were determined by regression analysis. Error bars show deviations from the line of best fit.

Graph 4.2.3 shows the mean of the scan values taken for each track, and plotted against the template DNA ratios. Linear regression analysis gave a correlation coefficient of 0.973 between template ratios, and final DNA concentrations of the two co-amplification products, showing a very strong positive correlation between initial template ratio of the co-amplificants, and the final ratio of the amplification products. This is obviously necessary if the competitive PCR system is to be used to estimate copy numbers of template DNA.

(iv). Competitive PCR in the presence of Foreign DNA.

The presence of large amounts of non-target DNA was thought to have a suppressive effect on the yield of specific product from PCR amplification. However it was not known whether this would affect the specific ratios of target to control produced in competitive PCR reactions. To investigate this, a competitive PCR reaction was carried out in the presence of a large amount of chicken genomic DNA, which had been checked to ensure that it produced no amplification product with the competitive PCR primers.

Materials and Methods.

PCR Conditions.

Six reactions were set up in duplicate, containing the amounts of target and non-target DNA per 20 µl reaction volume shown in Table 4.2.5, and amplified for 24 cycles.

Primers: DS49TOP, DS49PE, DSS.

Denature: 5 cycles x 3 min, 19 cycles x 1 min at 95°C.

Anneal: 65°C, 1 min. Polymerise: 72°C, 40 sec.

10 µl of the amplified product was electrophoresed on 2% agarose gel, and photographed. The negative was scanned by laser densitometry.

Table 4.2.5.

DNA Templates With and Without Foreign DNA.

Tube No.	AR10 Genomic DNA	pDSS Plasmid DNA	Chicken DNA
1	5 ng	0.2 ng	-
2	10 ng	0.2 ng	-
3	15 ng	0.2 ng	-
4	5 ng	0.2 ng	~ 1 mg
5	10 ng	0.2 ng	~ 1 mg
6	15 ng	0.2 ng	~ 1 mg

Results.

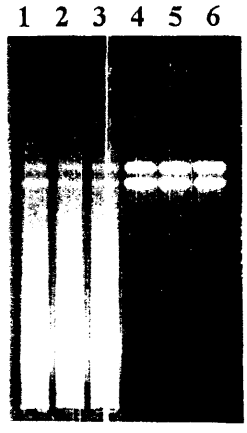
The photographed amplification products are shown in Figure 4.2.14. Densitometry results are shown in Table 4.2.6 and plotted against template DNA ratios in Graph 4.2.4.

Table 4.2.6.

pDSS Au mm	AR10 Au mm	AR10/pDSS
0.621	0.539	0.868
0.696	0.657	0.944
0.593	0.662	1.117
0.086	0.031	0.361
0.08	0.074	0.925
0.146	0.245	1.168

Figure 4.2.14.

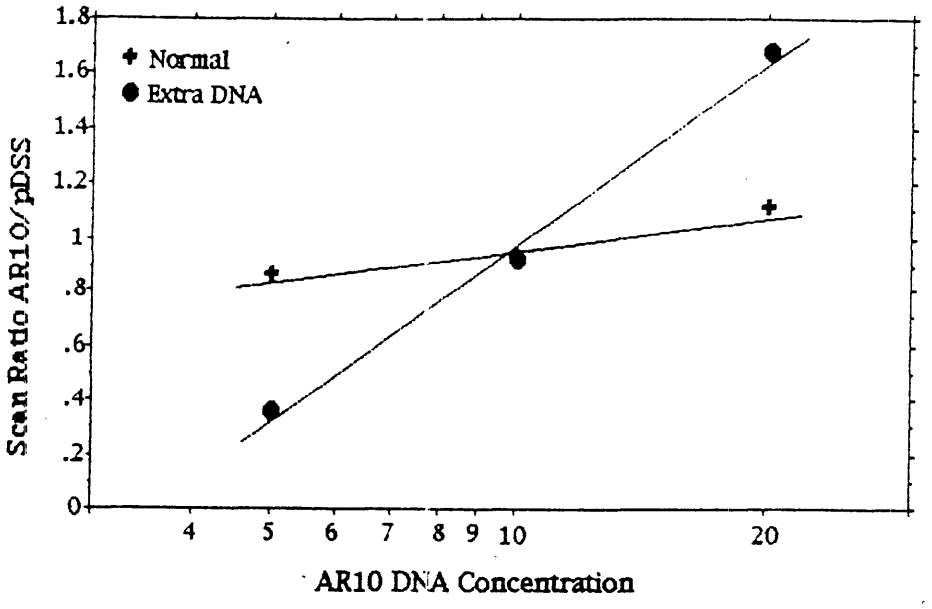
Co-amplification Products of AR10 and pDSS With and Without Excess Non-Target DNA



Lanes 1-3 show co-amplification products of 5,10,15 ng AR10 DNA and 0.2 ng pDSS DNA. Lanes 4-6 show co-amplification products of the same templates with ~ 1mg chicken DNA.

Graph 4.2.4.

Scan Ratios v Log Template DNA Concentration for Co-amplification Products of AR10 and pDSS With and Without Excess Non-Target DNA



The tabulated results show that the actual amount of product from PCR reactions with excess non-template DNA is considerably less than those without.

As can be seen from the graph, there is a linear relationship between template DNA ratios and log amplification product ratios in both cases, but the relationship is different. The greater slope of the PCRs with extra DNA could be due to the fact that these reactions obviously have lower amplification efficiencies than those without extra DNA, and therefore may still be in the exponential stage of the reaction, whereas those without extra DNA have reached the point where the presence of excess product has reduced the reaction rate. The figures from Table 4.2.5. demonstrate that although the individual scan results for target and control DNA were non-linear, the ratios of target DNA to log control DNA were linear. They also show that although the PCR reactions of the target and control DNA without excess non-target DNA were outside the exponential amplification stage, the plots of the ratios maintained linearity.

However the practical outcome of these result is that when competitive PCR is performed on templates containing large amounts of non-target DNA, e.g. rumen samples, standards for the competitive reactions should also contain equivalent amounts of non-target DNA, particularly if there is any likelihood of the amplifications proceeding beyond the exponential stage.

(v). Relative Amplification Efficiencies of Target and Control Sequences.

It was apparent from the previous experiments that the amplification efficiencies of target and control fragments were not exactly equal. By comparing ratios of target DNA and amplification products after a known number of cycles, it was possible to estimate the relative efficiencies. Since the densitometer scans only gave relative amounts of amplification product, it was not possible to measure absolute rates of amplification. Also, as with most enzyme catalysed reactions, the build up of product inhibits the reaction rate, at least partly due to product competition with primer at the annealing step. Therefore efficiency decreases as number of cycles increases.

Materials and Methods.

Target and control plasmid DNAs were co-amplified, with the concentration of control plasmid kept constant, and that of the target plasmid varied (see Table 4.2.7).

Amplification products were electrophoresed on acrylamide gel, autoradiographed, and scanned as for Experiment 4.2.2.2 (i.i).

Since the concentration of control plasmid DNA template was kept constant, target plasmid DNA amounts were directly plotted against the scan ratio of amplification products.

Table 4.2.7.

PCR Conditions for Estimation of Amplification Efficiencies.

Control DNA	Target DNA	Primers	AnnealingTemp.	Cycles
pJD6, 0.05ng	pJW4 0.025ng	G3A*, G2D2, G3I	70^0C	15
"	pJW4 0.05ng	"	70^0C	15
"	pJW4 0.075ng	"	70^0C	15
"	pJW4 0.1ng	"	70^0C	15
pDSS 0.05ng	pDS49 0.04ng	DS49top*, DS49PE, DSS	65^0C	18
"	pDS49 0.06ng	"	65^0C	18
"	pDS49 0.08ng	"	65^0C	18
"	pDS49 0.1ng	"	65^0C	18

* Indicates primers labeled with γ^{32}P ATP

Results.

See Graphs 4.2.5(a) and 4.2.5 (b).

Scan results are given in Appendix 3, Tables A3.6 and A 3.8.

Regression analysis of the pDS49/pDSS plot gives y = 12.325x + 0.123

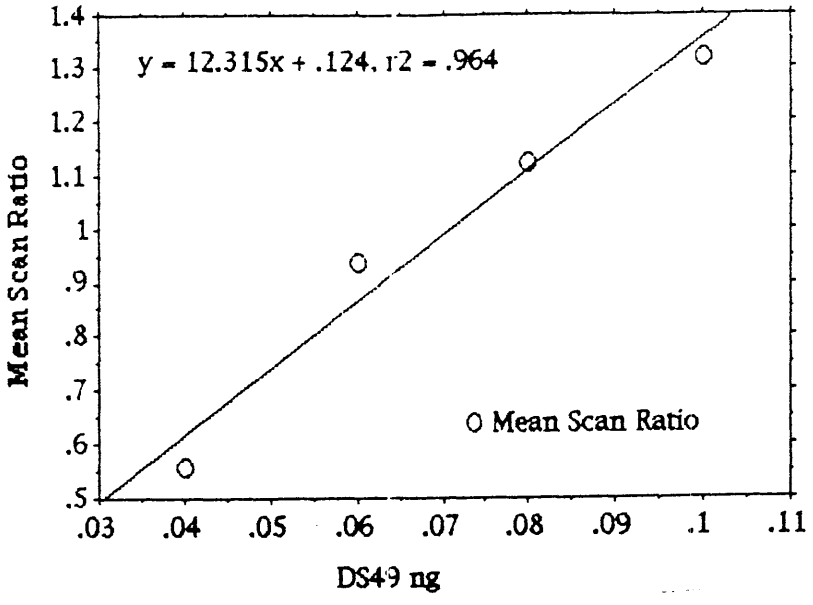
Regression analysis of the pJW4/pJD6 plot gives y=28.088x — 0.355

Where x = template concentration of target plasmid

and y = scan ratio of amplification products

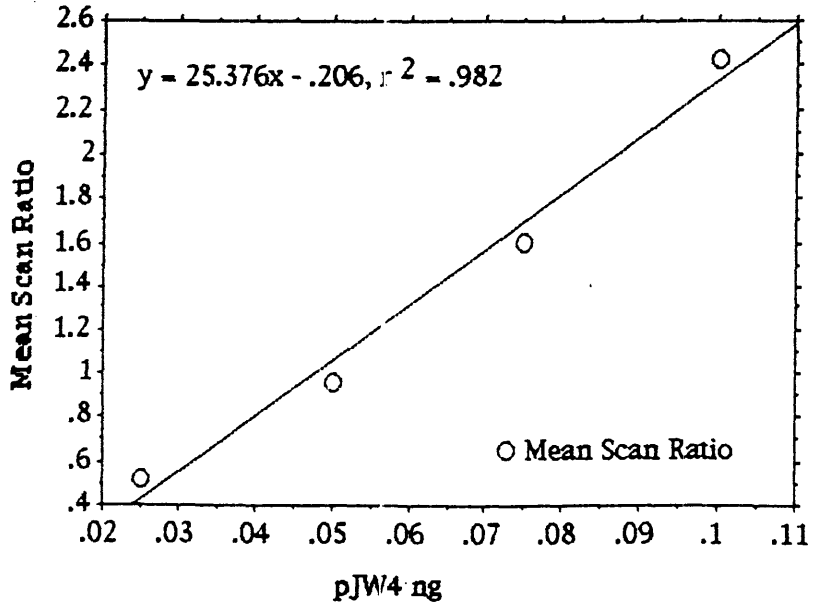
Graph 4.2.5(a).

Plot of Template DNA Amounts vs Scan Ratio of Amplification Products for pDS49 and pDSS.



Graph 4.2.5(b).

Plot of Template DNA Amounts vs Scan Ratio of Amplification Products for pJW4 and pJD6.



Calculations.

Amplification Equation.

If c = initial template copy number, and C = final copy number of amplification products,

and a = amplification efficiency,

then after 1 cycle of PCR, C =ac.

After 2 cycles of PCR, C = c + ac = c(a+1)

and after n cycles of PCR, $C = c(a+1)^{n-1}$ (Equation 1).

Amplification Efficiency of pDSS Relative to pDS49.

Template DNA for pDSS = 0.05 ng.

pDSS = 2940 bp, pDS49 = 5896 bp,

so for equal template copy numbers of target and control, when pDSS template = 0.05 ng

$$\text{template DNA for pDS49} = \frac{5986}{2940} \times 0.05 = 0.1 \text{ ng}$$

From regression analysis, when template DNA for pDS49 = 0.1 ng,

$$\text{scan ratio} = 12.326 \times 0.1 + 0.123 = 1.36$$

i.e. for pDS49/pDSS, when template ratio = 1, product ratio = 1.36

$$\text{But } C_{DS49} / C_{DSS} = \frac{c1(a1+1)^{17}}{c2(a2+1)^{17}} = 1.36 \qquad (n = 18)$$

and $c1/c2 = 1$ so $\frac{a1+1}{a2+1} = 1.36^{1/17}$ or $a1 + 1 = 1.36^{1/17} + 1.36^{1/17} a2$

Where a1 is amplification efficiency of pDS49,

and a2 is amplification efficiency of pDSS,

and c1 and c2 are template copy numbers of pDS49 and pDSS.

Taking a1 as 100% efficient relative to a2, then a1 = 1

$$\text{then } a2 = \frac{1+1}{1.36^{1/17}} - 1.36^{1/17} = 0.964$$

So pDSS amplification was 96.4% as efficient as pDS49 amplification.

Amplification Efficiency of pJD6 Relative to pJW4.

pJW4 = 4738 bp

pJD6 = 4599 bp

So for equal copy numbers, when pJD6 template = 0.05 ng

$$\text{pJW4 template DNA} = \frac{4738}{4599} \times 0.05 = 0.0515 \text{ ng}$$

From regression equation,

when pJW4 = 0.0515 ng, scan ratio = 28.088 x 0.0515 - 0.355 = 1.09

$$\text{C PJW4 / C PJD6} = \frac{(a1+1)^{15}}{(a2+1)^{15}} = 1.09$$

Taking a1 as 100%, then $a2 = \frac{(1+1)}{(1.09)^{1/15}} - (1.09)^{1/15} = 0.989$

So pJD6 amplification was 98.9% as efficient as pJW4 amplification.

Both amplification efficiency ratios were close enough to unity to cause no problems, even with large numbers of PCR cycles.

4.2.3 THREE PRIMER COMPETITIVE PCR FOR ESTIMATING TEMPLATE COPY NUMBERS.

The previous experiments had shown that the three primer competitive PCR system appeared to be a workable system for estimating copy numbers of target DNA, at least for *B. fibrisolvens* AR10 and *P. ruminicola* AR20. This section describes experiments using this system to estimate bacterial numbers, and bacterial genome size.

4.2.3(A) ESTIMATION OF BACTERIAL NUMBERS BY COMPETITIVE PCR.

Competitive PCR was performed on a diluted culture of heat killed *P. ruminicola* AR20, using pJD6 DNA as the control template. The number of bacteria in the culture was also estimated by plate counts of the same culture. Competitive PCR was also performed on DNA extracted from the culture to give some estimate of DNA extraction efficiency

Materials and Methods

P ruminicola AR20 was grown to stationary phase in 10 ml RF medium. Aliquots of the culture were serially diluted and spread on RF agar plates.

The remainder of the culture was heated to 100°C in a water bath. Cells were pelletted by centrifugation, washed twice in TE buffer, and finally suspended in 5 x original volume of distilled water.

As a comparison, competitive PCR was also performed on DNA extracted from 1 ml of the same culture and diluted to 2.5 ml.

Caesium chloride density gradient purified pJD6 DNA was used as control.

Standards of caesium chloride density gradient purified pJW4 DNA were amplified under the same conditions.

PCR conditions

Template: 1, 2 and 4 µl of washed AR20 cells suspended in 5 x original volume water;

1, 2 and 4 µl of AR20 DNA in 2.5 x original volume.

Standards: 0.025, 0.05, 0.075, 0.10 ng pJW4

All reactions contained 0.05 ng pJD6 control plasmid

Cycles:15

Denature: 10 x 5 min, 5 x 60s, @ 95°C;

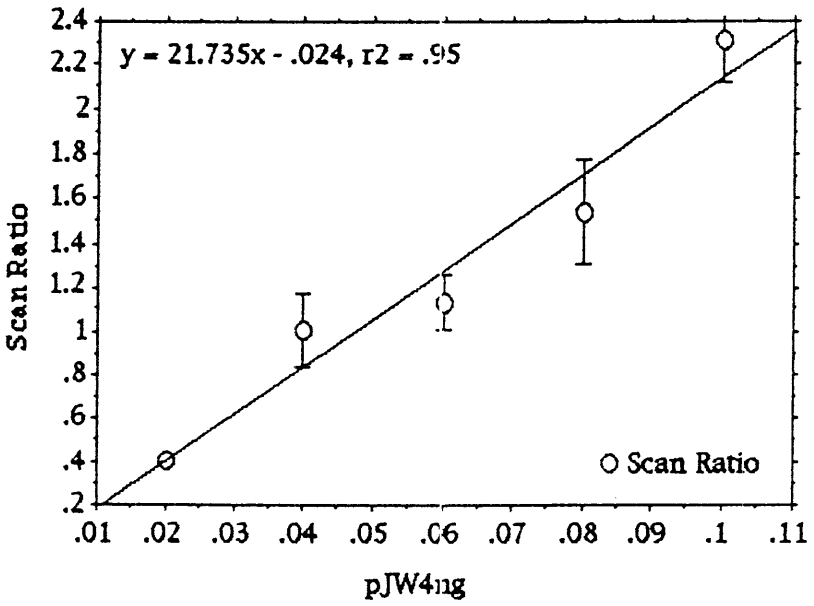
Anneal 40s, 65°C; Extension 40s, @ 72°C.

Primers: GA1B, G2D2, G3I (labeled with γ^{32}P ATP).

The amplification products were electrophoresed on 4% acrylamide gel, autoradiographed, and each band pair scanned once.

Graph 4.2.6.

Plot of Template Standards vs Scan Ratio for AR20 Bacterial Count.



Calculations.

The standards were plotted against the scan ratios, and regression analysis performed to give the slope of the line as: $y = 21.285 x - 0.015$

This was used to calculate unknowns as equivalent ng of pJW4

Copy number is given by: $\frac{\text{equiv. ng of pJW4}}{\text{weight of pJW4 DNA}}$

Where base number of pJW4 is 4738 bp

And weight of pJW4 DNA is $\frac{4738 \text{ X } 666}{6.03 \text{ X } 10^{23}}$ g $= 5.23 \text{ X } 10^{-6}$ pg.

Results.

For full scan results and calculations, see Appendix 3. The three methods were designed to measure different parameters, as shown below.

Count from competitive PCR of cells was $24.8 \pm 3.7 \text{ x } 10^8$ cells /ml

Count from competitive PCR of DNA was $9.3 \pm 1.1 \text{ x } 10^8$ genomes /ml

Count from plates was $3.2 \text{ X } 10^8$ cfu /ml

4.2.3(B). GENOME SIZE OF AR10 AND AR20 BY COMPETITIVE PCR.

By performing competitive PCR using templates of known amounts of genomic DNA together with standard templates containing known copy numbers of a unique genomic sequence, it is possible to calculate the weight of genomic DNA per copy of the target sequence.

Materials and Methods.

For *B. fibrisolvens* AR10, templates were 10,15 and 20 ng genomic DNA tested in duplicate.

PCR conditions were as for pDS49/pDSS competitive PCR, with 0.05 ng pDSS DNA included in each reaction. Standards were run in duplicate.

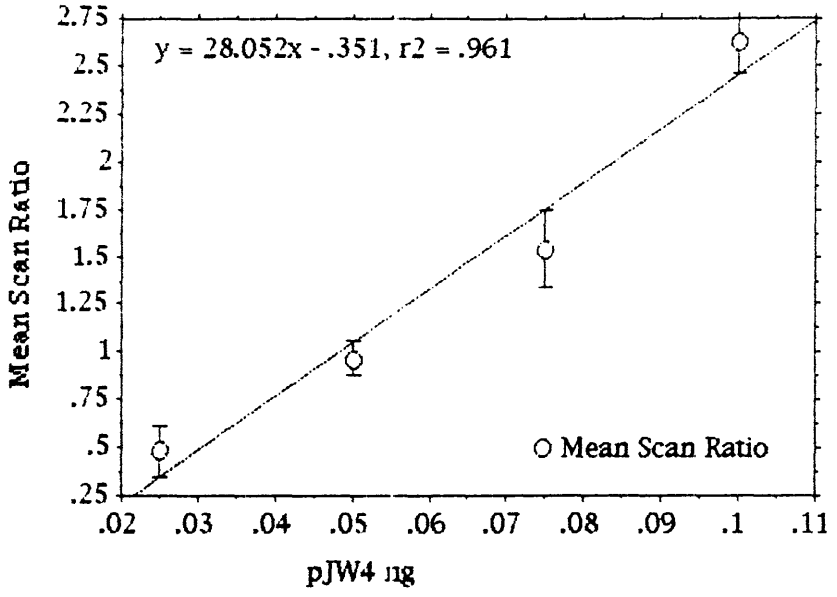
For *P. ruminicola* AR20, templates were 36, 48 and 60 ng genomic DNA. The 36 ng and 60 ng samples were amplified in duplicate.

PCR conditions and electrophoresis were as for pJW4/pJD6 competitive PCR, with 0.05 ng pJD6 DNA included in each reaction. Standards were run in duplicate.

Autoradiographs were taken of each set of reactions. Each band pair was scanned three times over slightly different tracks. Standard template DNA amounts were plotted against the scan ratios of the amplified products. The means for each genomic amplification product band ratio were calculated, and duplicates averaged.

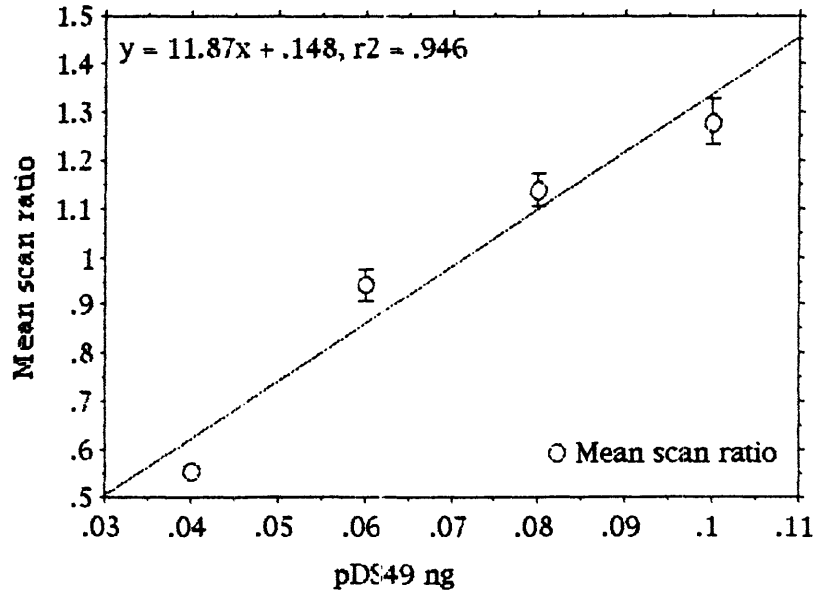
Graph 4.2.7.

Plot of Template Standards vs Scan Ratio for AR20 Genome Size.



Graph 4.2.8.

Plot of Template Standards vs Scan Ratio for AR10 Genome Size.



Calculations The standards were plotted against the mean scan ratios, (see Fig. 4.2.6 and 4.2.7) and regression analysis performed to give the slope of the line

For pDS49/pDSS: $y = 11.87x + .148$ ($r^2 = 0.964$).

For pJW4/pJD6: $y = 28.052x - 0.351$ ($r^2 = 0.961$).

From these, the equivalent ng of target was calculated for the unknown.

Genome size was given by:

$$\frac{\text{ng genomic DNA}}{\text{equiv. ng of target DNA}} \times \text{base no. of target DNA.} \qquad \text{Equation 2}$$

For pDS49, base no. = 5896 bp

For pJW4, base no. = 4738 bp

Results.

Full Scan results and calculations are given in Appendix 3. Tables 4.2.8 and 4.2.9 give summarised results.

Table 4.2.8.

P. ruminicola AR10 Genome Size.

AR10 ng	Mean Scan Ratio	Equiv. ng pDSS (from regression)	Genome Size Mb (from equation 2)	Mean, S.D., Std. Error
10.0	.052	0.031	1.54	Mean = 1.52
15.0	0.71	0.047	1.53	S.D. = 0.017
20.0	0.91	0.064	1.50	Std Error = 0.009
20.0	0.90	0.063	1.52	

Table 4.2.9.

B. fibrisolvens AR20 Genome Size.

AR20 ng	Mean Scan Ratio	Equiv. ng pJW4 (from regression)	Genome Size Mb	Mean, S.D., Std. Error
36.0	1.098	0.052	3.3	Mean = 3.47
48.0	1.446	0.065	3.51	S.D. = 0.15
60.0	1.868	0.079	3.9	Std Error =0.086.

Genome size of *B. fibrisolvens* AR10 = 1.52 $\pm$ 0.01 Mb

Genome size of *P. ruminicola* AR20 =3.47 $\pm$ 0.09 Mb

4.2.4. FOUR PRIMER COMPETITIVE PCR.

Because it had not proved possible to make deletant control plasmids for OB156 and AR29, it was decided to use controls similar to those of Kellogg *et al.* (1990), who used a section of foreign DNA, unrelated to the sequence to be amplified, as the control sequence. This control sequence was co-amplified with the target DNA using its own primer pair. The sequence chosen was a cloned piece of *Drosophila buzatii* alcohol dehydrogenase DNA, pJupiter, donated by D. Schaefer. Since this sequence was not of bacterial origin, it was unlikely to be present in rumen contents. Two primers, JUPF and JUPI were selected from the sequence to give a 441 bp amplification product (see Appendix 1).

(i) Four Primer Competitive PCR of *B. Fibrisolvens* OB156.

Competitive PCR reactions were set up to investigate the four primer system, and to determine the linearity of a set of standard templates.

Co-amplification reactions were set up using counted OB156 cells diluted in rumen samples as target template, and fixed amounts of pJupiter as control template. (Because OB156 cells do not form chains like *P. ruminicola* AR20 cells, and in some cases *B.fibrisolvens* AR10 cells, a direct count of cells was possible). The forward primers for both target and control, GOBF4 and JUPF were labeled with γP^{32} ATP. Amplification products were electrophoresed on both agarose and acrylamide gels.

Materials and Methods.

Competitive PCR standards were set up according to Table 4.2.10

PCR conditions.

Denature: 95°C, 10 cycles 5 min, 20 cycles 1 min.

Anneal: 65°C, 30 cycles 40 sec

Polymerise: 72°C, 30 cycles 40 sec.

Reaction blanks consisted of: 1. Reagents + rumen sample + control plasmid

2. Reagents + rumen sample

3. Reagents

Table 4.2.10.

Template DNA for Four Primer Competitive PCR

Tube No.	Template	pJupiter ng.
1 & 2	2x OB156 cells	2×10^{-6}ng
3 & 4	5x OB156 cells	2×10^{-6}ng
5& 6	10x OB156 cells	2×10^{-6}ng
7& 8	25x OB156 cells	2×10^{-6}ng
9 & 10	100 x OB156 cells	2×10^{-6}ng
11	Rumen Blank	2×10^{-6}ng
12	Rumen Blank	-
13	Water Blank	-

After amplification, 2 µl from each reaction was loaded onto a 4% acrylamide gel, and 7 µl onto 2% agarose gel. The agarose gel was photographed and the acrylamide gel autoradiographed, and negative and autoradiograph were scanned with the laser densitometer. Scan ratios were plotted against template copy number for the autoradiograph, and log copy number for the negative of the agarose gel, to obtain linear plots.

(ii). Determination of OB156 Numbers in Rumen Samples.

The four primer competitive PCR system was used to determine the number of OB156 cells in rumen samples.

Materials and Methods.

Five rumen samples, taken from a sheep which had been inoculated with OB156, were prepared as described in chapter 4 [4.2.5.(v). page 4.36]. These were co-amplified with pJupiter, as for the four primer standard curves above, with counted OB156 cells diluted in rumen samples as standards. Template DNAs were as shown in Table 4.2.11, and PCR conditions were as for the preparation of standard curves (4.2.4. (i) above).

Table 4.2.11.

Templates for Estimation of OB156 in Rumen samples.

Tube No.	Template	pJupiter ng.
1	2x OB156 cells	2×10^{-6}ng
2	5x OB156 cells	2×10^{-6}ng
3	10x OB156 cells	2×10^{-6}ng
4	M1	2×10^{-6}ng
5	M2	2×10^{-6}ng
6	M3	2×10^{-6}ng
7	M4	2×10^{-6}ng
8	M5	2×10^{-6}ng
11	Rumen Blank	2×10^{-6}ng
12	Rumen Blank	-
13	Water Blank	-

After amplification, 2 µl of the products were electrophoresed on 4% acrylamide gel, and 7 µl on 2% agarose gel. Gels were photographed or autoradiographed as above. Scan ratios of the standards were plotted against template copy numbers of the standards. The slopes of the lines were determined by regression analysis, and copy number of the unknowns calculated.

(iii). Comparison of Competitive and Non-Competitive PCRs.

Since the amount of pJupiter DNA was the same in all reactions, its effect on the amplification of OB156 DNA could be assumed to be constant in all reactions. Therefore valid comparisons could be made between the amounts of OB156 DNA produced in each reaction.

The same results as for 4.2.4 (ii), above were used, but the scan readings for the pJupiter controls were ignored, and only the scan readings from OB156 standards and unknowns were considered. The OB156 densitometer scan readings from the standard curve were plotted against template copy numbers of OB156. OB156 cell numbers were determined from OB156 scan readings of the five unknown rumen samples from the same negative.

Results.

(i). Four Primer Competitive PCR of OB156.

Results of the densitometer scans of the competitive PCRs are shown in tables 4.2.12 and 4.2.13; the scan ratios from the autoradiograph are plotted against template copy numbers in graph 4.2.8.; the scan ratios from the Polaroid negative are plotted against the logarithms of the template copy numbers in graph 4.2.9..

It can be seen that there is a linear relationship between the number of OB156 cells in the template for the autoradiograph, and the logarithm of cell numbers in the Polaroid negative, and the ratio of amplification products. The results in both cases gave very high correlation coefficients. This suggested that the four primer competitive PCR system would provide a reliable method for estimating template copy numbers .

Table 4.2.12.

Polaroid Negative Scans of OB156/ pJupiter Four Primer Competitive PCR.

OB156 Cell Nos	OB156 Scan Reading	pJupiter Scan Reading	Scan Ratio OB156/ pJup
2	0.05	0.571	0.105
5	0.272	0.726	0.376
10	0.306	0.549	0.557
25	0.510	0.573	0.89
100	0.812	0.615	1.32

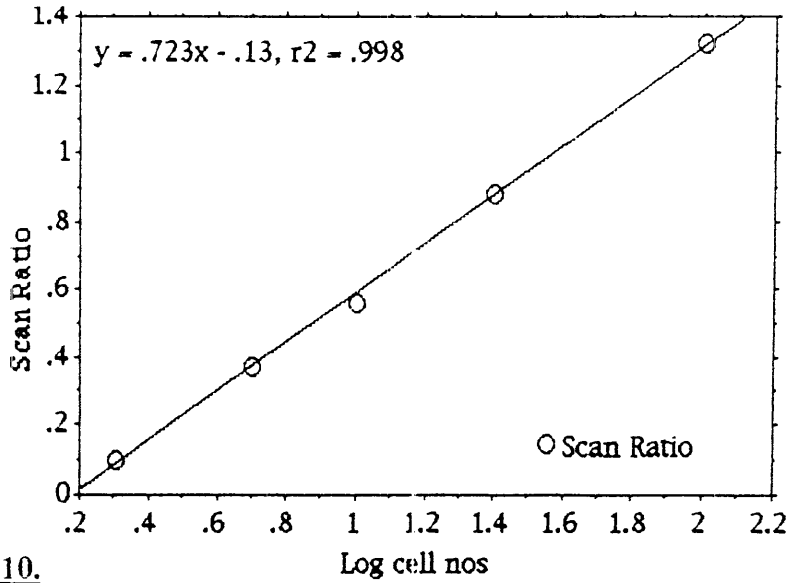
Table 4.2.13.

Autoradiograph Scans of OB156/ pJupiter Four Primer Competitive PCR.

OB156 Cell Nos	OB156 Scan Reading	pJupiter Scan Reading	Scan Ratio OB156/ pJup
2	0.161	0.206	0.782
5	0.758	0.459	1.65
10	0.746	0.293	2.57
25	2.981	0.643	4.64
100	5.421	0.388	13.97

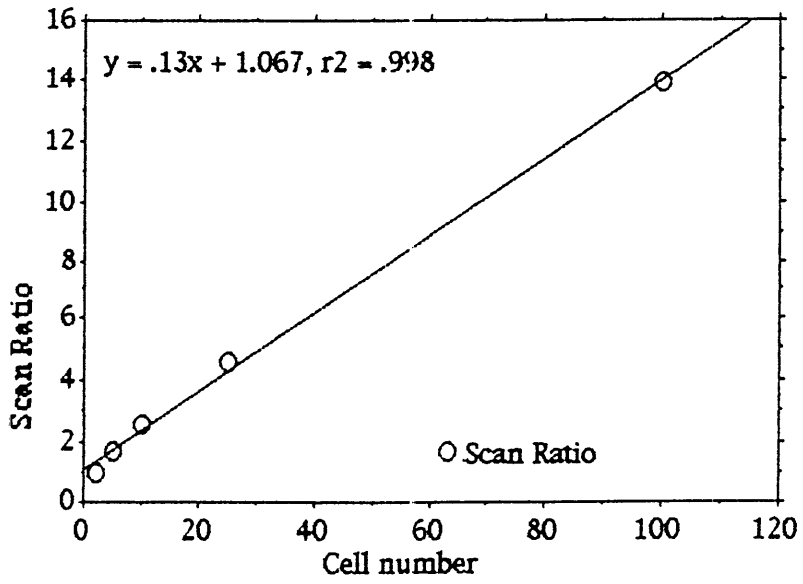
Graph 4.2.9.

Ratio of Scan Readings, OB156/pJupiter - Polaroid Negative.



Graph 4.2.10.

Ratio of Scan Readings, OB156/pJupiter - Autoradiograph



(ii). Determination of OB156 Numbers in Rumen Samples.

Scans of the OB156 standards from the Polaroid negative and the autoradiograph are given in tables 4.2.14 and 4.2.15. Plots of scan results against log template cell numbers and cell numbers are given in graphs 4.2.11. and 4.2.12. Scan results and copy numbers of OB156 in the unknown rumen samples are given in table 4.2.8.

Table 4.2.14.

Polaroid Negative Scans of OB156 standards.

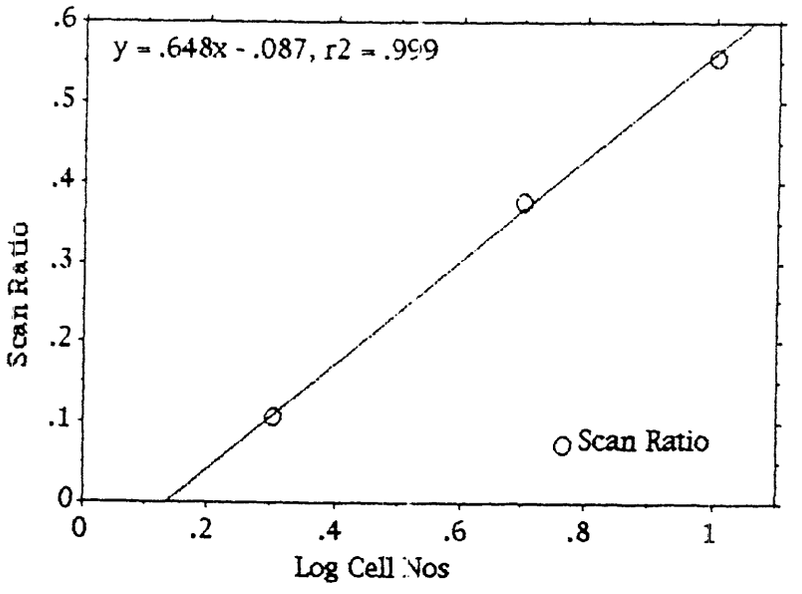
OB156 Cell Nos	OB156 Scan Reading	pJupiter Scan Reading	Scan Ratio OB156/ pJup
2	0.05	0.571	0.105
5	0.272	0.726	0.376
10	0.306	0.549	0.557

Table 4.2.15.
Autoradiograph Scans of OB156 standards.

OB156 Cell Nos	OB156 Scan Reading	pJupiter Scan Reading	Scan Ratio OB156/ pJup
2	0.161	0.206	0.782
5	0.758	0.459	1.65
10	0.746	0.293	2.57

Graph 4.2.11.

Standard Curve for OB156 Numbers - Polaroid negative.



Graph 4.2.12.

Standard Curve for OB156 Numbers - Autoradiograph.

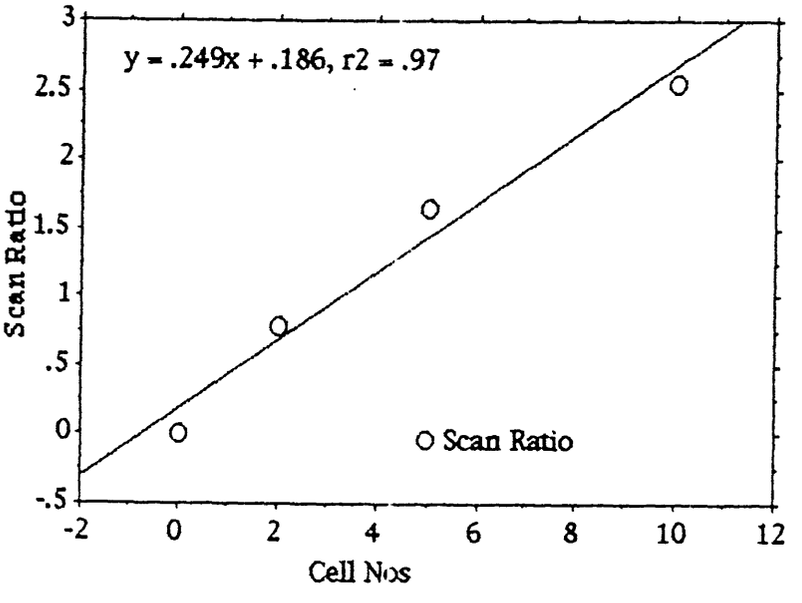


Table 4.2.16.

Estimated Cell Numbers from Polaroid Negative and Autoradiograph.

	Scan Ratios		Cell Numbers	
Unknown Sample	Negative	Autorad	Negative	Autorad.
M1	0.54	1.75	9	7
M2	0.19	0.1	3	1
M3	0.47	2.04	7	7
M4	0.118	0.33	2	1
M5	0.08	-	2	-

Cell numbers are per microlitre of rumen sample.
Cell number estimates are given to nearest whole number.

Calculated numbers of OB156 cells/μl rumen sample differ slightly between the autoradiograph estimation and the Polaroid negative estimation. Since both sets of results are from the same amplification reactions, the differences must be due to errors of measurement.

(iii). Comparison between Competitive and Non-Competitive PCRs.

Graph 4.2.13 shows the plot of OB156 scan readings against the logarithm of template copy numbers. Table 4.2.17. shows the estimates of cell numbers from both competitive and non-competitive PCRs. A comparison of Graphs 4.2.11 and 4.2.13 shows that the competitive PCR plot approaches linearity more nearly than the non-competitive plot. The effect of the use of the co-amplification ratio is to produce a smoother curve, with a higher correlation coefficient between template (cell) numbers and scan results. However the difference in cell numbers estimated was small.

Graph 4.2.13

Standard Curve for Non-Competitive PCR Estimation of OB156 Numbers.

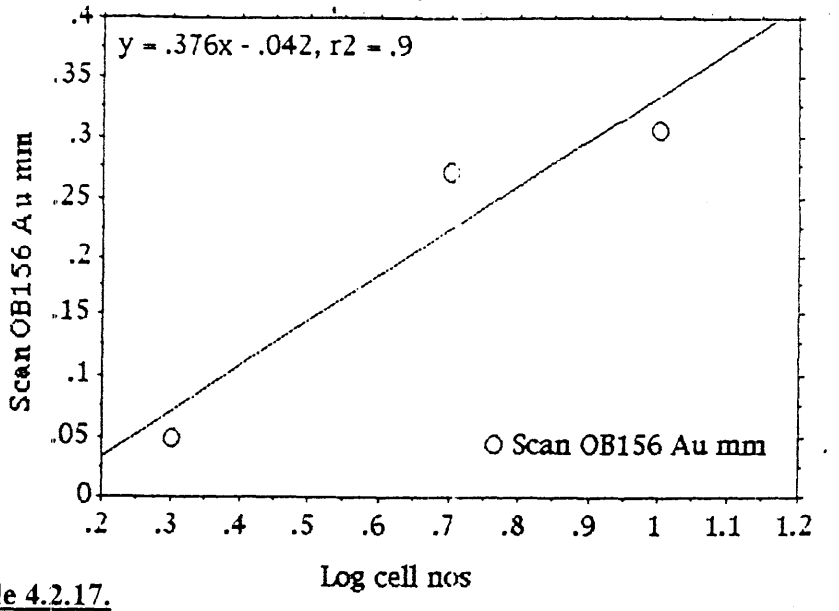


Table 4.2.17.

Comparison of Cell Number Estimation from Competitive and Non-Competitive PCR.

			Cell Numbers	
Unknown Sample	Scan Ratio	Scan OB156	Competitive PCR	Non-Competitive PCR
M1	0.54	0.352	9	11
M2	0.19	0.105	3	2
M3	0.47	0.299	7	8
M4	0.118	0.105	2	2

Cell numbers are per microlitre of rumen sample.
Cell number estimates are given to nearest whole number.

As can be seen from Table 4.2.17, the estimates of OB156 numbers are very similar, whether derived from competitive or non-competitive PCR of the same samples.

Correlation Between Different Methods of Estimating Cell Numbers.

The correlation coefficient between numbers estimated from the autoradiograph and the Polaroid negative was 0.924.

The correlation coefficient between numbers estimated from the competitive and non-competitive estimations from the Polaroid negative was 0.987.

These figures show high correlation between all methods of estimating cell numbers in this experiment.

Table 4.2.18.

Means and Standard Deviations of All Estimates of OB156 Numbers.

Sample no.	Counts	Mean	Std. dev.	Std. error
M1	9,7,11	9	2	1.1
M2	3,1,2	2	1	0.6
M3	7,7,8	7	0.6	0.3
M4	2,1,2	2	0.6	0.3
M5	2,1	1.5	0.7	0.5

Table 4.2.18 shows that all sampling methods give good approximations to each other's results, and suggest that there is a maximum difference of between $\pm$ 1 and $\pm$ 2 in estimating numbers of cells in each sample from the different methods .

PART 3. DISCUSSION.

The problems with heteroduplex formation in the two-primer competitive PCR system, and the consequent decision to abandon the two primer system and develop an improved competitive PCR system have already been discussed at the end of part 4.2.1 and the beginning of part 4.2. of this chapter.

The three primer modification of the competitive PCR system by the use of a common primer, and primers specific for target and control proved a successful and robust system for quantitating PCR, eliminating the heteroduplex problems of the earlier two primer system. A considerable advantage of the three primer system was the ability to select the specific control primer from known sequence, and use it to produce the deletant control sequence. The enzymic method for generating deletions within a DNA fragment is rendered considerably more complex if no unique restriction site is available. One possible solution is to use an enzyme that cuts the plasmid at very few sites. Then linearised plasmid fragments can be selected from partial enzymic digests, and the correct product determined. However, where DNA sequences are difficult to clone, as is the case with much rumen bacterial DNA, the enzymic method has the advantage of producing a range of "nested" deletants, thus increasing the chances of successful cloning.

The difficulties with the three primer method were due to the difficulties of cloning rumen bacterial DNA in *E. coli*. (Gregg,1992). In some cases where there was no suitable unique site for enzymic deletion, it proved impossible to clone the DNA fragments carrying the PCR generated deletions, and no control sequence was produced for OB156 and AR29. This problem could possibly be overcome by the selection of several deletion primers that produced slightly different deletion sequences. As can be seen from Appendix 1, the deletion fragments from the deletion primers DSS and DSG differed by only three base pairs in one half of the primer, but it was possible to clone the DS49top-DSS fragment, albeit at a very low success rate, and not possible to clone the DS49top-DSG fragment.

The four primer competitive PCR method had originally been considered a less attractive alternative for two reasons. First because it was feared that totally unrelated DNA fragments might have widely differing amplification efficiencies. Second because it was foreseen that it would be difficult to radio-label two different primers equally. However, while relative amplification efficiencies were not investigated, the method was shown to produce satisfactorily linear standards, and was used to estimate OB156 bacterial numbers in a series of rumen samples.

The advantage of the four primer system over the three primer system is that the same control sequence can be used with a number of different target sequences, thus obviating the need to produce a new control sequence for each target sequence. The main disadvantage is that two different primers must be labeled for each co-amplification. This is not only more expensive, but unequal labeling of the two primers may skew the measurements of the product ratios. This problem of unequal primer labeling is, of course, only relevant when the products are determined by autoradiography. Both systems appeared to work well for estimating template copy numbers of unknowns.

When the competitive PCR system was used to estimate AR20 cell numbers, it produced a result eight times that of the number estimated by plate count. There could be several contributing causes to this result. First, a stationary phase culture was used, and as the age of the culture increases, AR20 cells tend to develop into chains. Each of these chains gives a single c.f.u., but contains several complete cells, each of which will be counted by PCR. Second, a cell in the process of division may possess two copies of at least part of the chromosomal DNA. Third, plate culture will count only viable cells, whereas PCR counts any cell, alive or dead, whose DNA remains sufficiently intact for the target sequence to retain its integrity. However, since AR20 contains extremely active DNAses, this third factor is not likely to be of much significance. Fourth, most cultures contain a proportion of viable, but non-culturable cells, which for undetermined reasons do not show up on plate counts. (Pickup, 1991). Therefore it is well known that plate

counts will frequently underestimate the number of cells in a culture compared with direct counts, and it is probable that PCR may overestimate the number of living cells in a culture.

The comparison between live cells and extracted DNA suggested that only about 40% of total genomic DNA was extracted from the bacterial cells.

Competitive PCR provides a simple method of genome size estimation. The genome sizes estimated fall within the range of known bacterial genome sizes. *E. coli*, a gram negative rod, morphologically similar to *P. ruminicola* has a genome size of 3.84 Mb (Stanier *et al,* 1987), compared to 3.5 Mb estimated for *P. ruminicola. Desulfovibrio,* an obligately anaerobic motile rod, like *B. fibrisolvens*, has a genome size of 1.65 Mb (Stanier *et al,* 1987) compared to 1.5 Mb estimated for *B. fibrisolvens.*

Comparisons of autoradiograph and Polaroid negative for estimating amounts of amplification product confirm that the densitometry scan of the autoradiograph gives a linear relationship between template copy number and DNA produced as measured by laser densitometry; whereas the relationship is logarithmic with the Polaroid negative. This has already been considered in the Discussion section of Chapter 3. Thus the autoradiograph is the more sensitive measure. However it is a considerably simpler procedure to electrophorese the amplified products on agarose gel and photograph the results than to electrophorese the products on an acrylamide gel and autoradiograph the results. Moreover, with agarose gels labeled primers are not rquired. So in estimations where accuracy is of major importance, as in estimation of genome size, autoradiography of radiolabelled amplification products is preferable. However, for following changes in bacterial numbers in rumen samples, the simpler method of photographing ethidium bromide stained agarose gels should give adequate results, and has the advantage of not requiring the use of radioactive materials. As previously discussed, digital scanning of ethidium bromide stained agarose gels may give a linear relationship between template

copy number and ethidium bromide fluorescence, which would improve the sensitivity of the agarose gel method.

The four primer method was used to estimate numbers of OB156 cells in unknown rumen samples taken from sheep, which had been inoculated with OB156 pBHermF some days before the M1 sample, was taken. Unfortunately it was not possible to use the samples from the multiple tracking experiment (Chapter 3, section 3.2.5). These samples had been stored at -20°C for about 18 months from the end of the experiment, and it proved impossible to obtain OB156 PCR amplification product (or any other specific bacterial amplification product) from these samples. It was not known whether this was due to refrigerator failure at some time during the storage period, or simply because the DNA in the samples was not stable at this temperature for long periods.

Comparison between graphs of competitive PCR and non-competitive PCR as methods for estimating template copy numbers shows that competitive PCR undoubtedly produces more linear, and hence more accurate results. However, statistical analysis shows a high correlation between the result from the two methods. It to would seem to be a considerable overstatement to say that non-competitive PCR is not quantifiable. With care in loading exactly equal amounts of amplification product on to the electrophoresis gel reasonably linear standard curves can be produced, and reproducible results obtained. This is shown well in the results of the duplicate reactions shown in Table 3.2.3 in the previous chapter. It should be emphasised that the results were obtained using a waterbath thermocycler, which gives very uniform temperatures across all samples. Whether block thermal cyclers have the same degree of uniformity of temperature across all samples is not known.

Thus it appears that the use of competitive PCR, and particularly the use of radiolabelled primers for autoradiography will indeed give the most accurate and sensitive estimation of template copy number. But where highly accurate results are not required,

the use of competitive PCR is not necessary. With care, it has been shown possible to get good estimations of template copy numbers from ordinary PCR reactions.

The ability of PCR techniques to enumerate bacteria in samples containing very high levels of non-target bacteria has potential use, not only in the rumen environment, but in other environments such as soils, sewage, faecal material, foods, water, where in may be necessary to enumerate specific bacteria. Possible uses include quantitating genetically modified organisms in such samples, enumerating potential pathogens in water supplies or food. As well, the techniques have application in ecological studies for determining populations of naturally occurring strains of bacteria. There is no reason to suppose that the PCR quantitation methods would be any less successful if applied to eucaryotic microorganisms or viruses.