

FINAL REPORT on FSA project G03031

APPENDICES

Appendix 1: Genomic DNA extraction from rumen fluid using the CTAB protocol:

3.1 Additional reagents required:

CTAB Buffer			
Reagent	Initial Conc.	Amount Added	Final Conc.
CTAB	-	2.0 g	2% m/v (54.9 mM)
Tris-HCl (pH = 8.0)	1 M	10 ml	100 mM
EDTA (pH = 8.0)	0.5 M	4 ml	20 mM
NaCl	5 M	28 ml	1.4 M
PVP	-	1 g	1% m/v (22.8 mM)
Adjust pH to 5.0 with HCl and make up to 100 ml with dH ₂ O			

TE Buffer 10 mM Tris-HCl (pH = 8.0), 1 mM EDTA (pH = 8.0)

- 3.2 Add 20 mg of ground maize, soybean or oilseed rape seeds and 1 ml of rumen fluid in a fresh 2 ml microcentrifuge tube. (or 50 mg of freeze-dried material)
- 3.3 Add 900 µl of CTAB buffer (pre-warmed at 65.0 °C) to each sample and incubate at 65.0 °C for 60 min.
- 3.4 Centrifuge sample at 12,000 g for 5 min and transfer the supernatant in a fresh 2 ml microcentrifuge tube.
- 3.5 Add 900 µl of Phenol: Chloroform : Isoamyl Alcohol (25:24:1) to each sample and mix thoroughly.
- 3.6 Centrifuge each sample at 12,000 g for 15 min at 4.0 °C.
- 3.7 Carefully transfer the upper layer to a fresh 2 ml microcentrifuge tube.
- 3.8 Add 900 µl of Phenol : Chloroform : Isoamyl alcohol to each sample and mix thoroughly.
- 3.9 Centrifuge each sample at 12,000 g for 10 min at 4.0 °C.
- 3.10 Carefully transfer the upper layer to a fresh 2 ml microcentrifuge tube.
- 3.11 Add 900 µl of Chloroform : Isoamyl alcohol (24:1) to each sample and mix thoroughly
- 3.12 Centrifuge each sample at 12,000 g for 10 min at 4.0 °C.
- 3.13 Carefully transfer the upper layer to a fresh 1.5 ml microcentrifuge tube.
- 3.14 (Optional): If RNase treatment is needed, add 3 µl of RNase A (10 mg/ml) in the sample and incubate at 37.0 ° for 15 min - 1 h.
- 3.15 Add 50 µl of 7.5 M Ammonium Acetate and 450 µl of isopropyl alcohol to each sample and incubate at -20.0 °C for 1 h (samples can be incubated in this solution up to a month at -20.0 °C).
- 3.16 Centrifuge samples at 7,500 g for 15 min at 4.0 °C.
- 3.17 Decant the supernatant carefully so that the DNA pellet is not disturbed.
- 3.18 Add 1 ml of 70% ethanol to each DNA pellet and mix by carefully inverting the tube.
- 3.19 Centrifuge samples at 7,500 g for 5 min at 4.0 °C and discard the supernatant.
- 3.20 Repeat steps 3.18-3.19 twice.

- 3.21 Carefully decant the ethanol making sure that the DNA pellet is not disturbed. Air-dry the DNA pellets at room temperature (about 15 min).
- 3.22 Re-suspend each DNA pellet in 50-200 μ l of TE buffer. (depending on minimum concentration needed).
- 3.23 Incubate DNA samples at 85.0 $^{\circ}$ C for 10 min so that any DNase activity will be diminished. Moreover, if the DNA pellet has not been fully re-suspended, gently flick the tube twice during that incubation. (If the DNA pellet is still not completely dissolved, incubate samples on ice and flick each tube gently every few minutes till the DNA is completely dissolved or leave at a shaking platform at 4.0 $^{\circ}$ C overnight).

Appendix 2: Standard Operating Procedure for qPCR amplification of endogenous and transgenic DNA from samples extracted from rumen fluid.

Hazards:

Not Applicable

Protection:

Laboratory coat, gloves

Waste:

Not Applicable

Spill Clean-Up:

In order to avoid cross-contamination of samples, all pipette tips used in these protocols were lined with filter material and were certified DNase- and RNase-free. Moreover, benches and re-usable plastic and glassware were disinfected with 10% Hypochlorite solution.

Procedure:

Set-up Reactions

Set-up Reaction Notes:

- Set-up reactions in special qPCR set-up area.
- Keep reagents on ice.
- Make reaction cocktail fresh prior to the amplification.
- Use white 96-well plates (Thermo Scientific, Epsom, UK).
- qPCR amplifications were performed using the Opticon 3 system (MJ Research Technologies, UK)
- Primer concentration was normalised to 10 μM .
- Template DNA was diluted to a final concentration of 10 $\mu\text{g}/\mu\text{l}$ in 5 $\mu\text{g}/\text{ml}$ Herring Sperm DNA (Promega, Southampton, UK).
- Real-time PCR reaction mixtures were prepared using the SYBR Green JumpStart ReadyMix (Promega Southampton, UK), according to the manufacturer's instructions. In brief, each qPCR cocktail contained the following:

Reaction Mixture:

7.5 μl SYBR Green Mix (2x)
1 μl template DNA (10 $\text{ng}/\mu\text{l}$)
1 μl Forward primer (10 μM)
1 μl Reverse primer (10 μM)
4.5 μl H₂O (DNase-free)

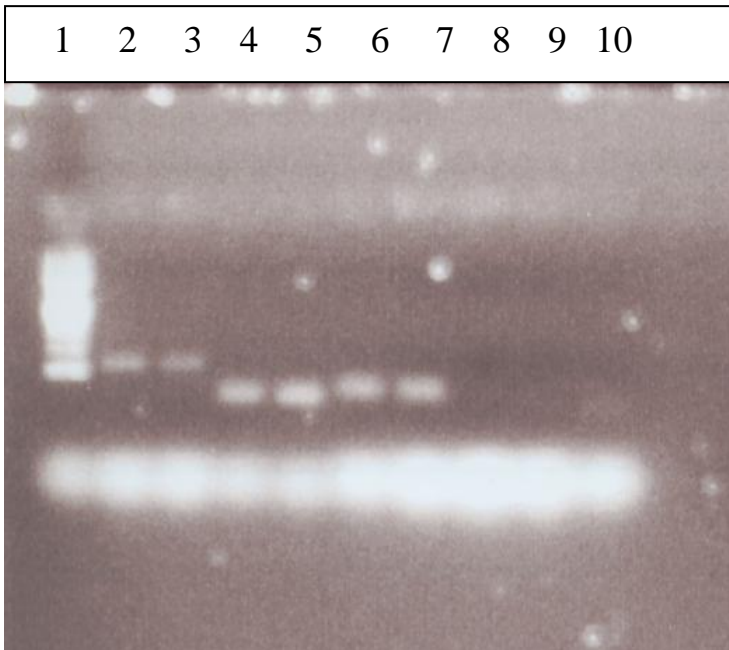
Amplification was performed according to the following protocol:

1. Initial Denaturation at 95 °C 4 min
2. Denaturation at 95 °C 30 sec
3. Annealing (look table 1 for temperature and time specific for each primer pair)
4. Elongation at 72 °C 30 sec
5. Plate Read
6. Number of cycles (steps2-5, Table 1)
7. Melting Curve from 65-95 °C, Read every 0.2 °C.

Following amplification:

1. After PCR is finished, remove the plates from the machine.
2. Amplification of the expected fragments was evaluated by analysis of the melting curve produced in each amplification reaction.
3. Standard curves were constructed in each plate by 5 serial dilutions of genomic plant DNA (dilution factor : 10).
4. Quantification of each sample was performed by correlating respective C(t) values with the results obtained from the standard curves present in each plate.
5. Relative quantification was performed by utilising the ddC(t) ratio of each transgene over the equivalent housekeeping gene.
6. Data analysis was performed in GENSTAT by repeating measurements ANOVA of the average values obtained for each biological replicate.

Appendix 3 – Amplification of housekeeping and transgenes in DNA extracted from GM plants by PCR



Amplification of Maize genes

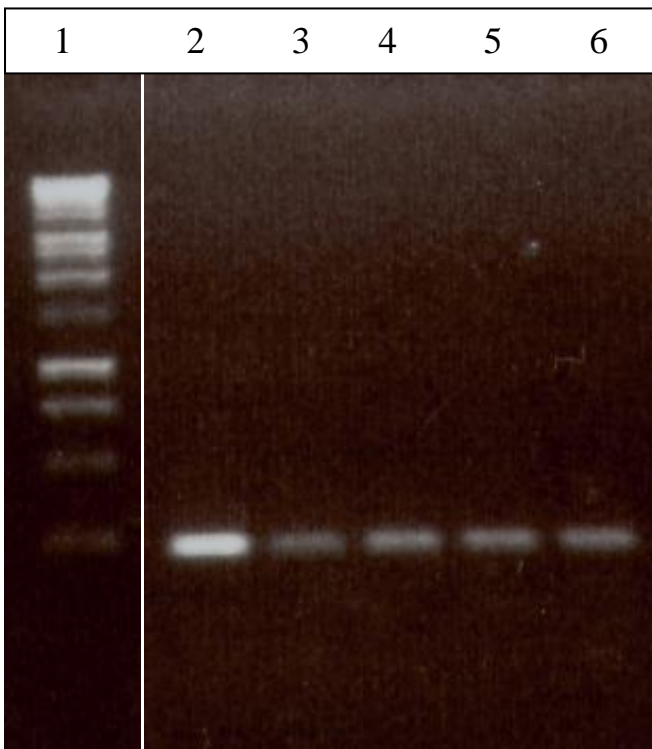
Lane 1 – 100bp DNA ladder

Lanes 2,3 – 18S rDNA, primers, TR03 + TR04

Lanes 4,5 – Zein housekeeping gene, primers Zenm3 + Zenm1

Lanes 6,7 – Cry1a GM DNA, primers Cry1F F+ Cry1F R

Lanes 8,9,10 – negative controls for each primer pair



Optimisation of amplification conditions for soya lectin (housekeeping) gene

Lane 1 100bp DNA ladder

Lanes 2-6 – lectin gene, primers LecF + LecR at 62°C, 60°C, 58°C, 56°C and 55°C respectively

1 2 3 4 5 6 7 8



Amplification of plant DNA with different primer pairs

Lane 1 – primers BarF1 and R1*

Lane 2 – primers NPTII F1 and RI

Lane 3 – primers Bars F1 and R3

Lane 4 – primers Bars F3 and R3

Lane 5 – primers Barn F2 and R1*

Lane 6 – primers Cry1A F3 and R3

Lane 7 – primers IvrF and R*

Lane 8 – primers CruF and CruR*

*- successful amplification

Appendix 4**Analytical Laboratory Results**

Requested by :-

Kostas Minas

Date :- 23/04/10

Samp. No.	D.M. g/Kg	MOISTURE g/Kg	N g/Kg	C.P. g/Kg	ADF g/Kg	ADL g/Kg	NDF g/Kg	ASH g/Kg	O.M. g/Kg	E.E. g/Kg
6a			16.96	106.00	222.41	29.62	450.76	74.31	925.69	18.65
6b	848.89	151.11	17.03	106.44	217.53	28.94	432.11	74.94	925.06	19.08
6c			17.53	109.56	219.72	29.43	462.80	75.64	924.36	20.99
7a			17.69	110.56	98.11	15.08	224.56	52.85	947.15	24.16
7b	854.02	145.98	18.16	113.50	98.20	15.19	209.09	52.28	947.72	23.56
7c			17.06	106.63	102.00	15.05	200.44	51.54	948.46	23.77

Appendix 4 - Analytical Laboratory Results for the High and Low Concentrate Diets used in the RUSITEC trials.

All values corrected to 100% Dry Matter (D.M)

Sample 6 – low concentrate diet

Sample 7 – high concentrate diet

Abbreviations:

- N - Nitrogen
- C.P. – Crude Protein
- ADF – Acid Detergent Fibre
- ADL – Acid Detergent Lignin
- NDF – Neutral Detergent Fibre
- ASH – Total Mineral Content
- O.M. – Organic Matter
- E.E. – Ether Extract

Appendix 5 - tRFLP analysis of microbial populations

PCR

	Per reaction (ul)
Molecular water	11.75
PCR buffer (5x)	5.0
MgCl ₂ (25mM)	1.75
dNTPs (10mM)	0.5
Forward primer (50uM)	0.25
Reverse primer (50uM)	0.25
Taq polymerase (5u/ul)	0.5
DNA template (150ng) + molecular water	5.0
Final volume	25.0

For universal bacteria analysis, 27F and 1389R used with one with fluorescent tag.
PCR program for 27F (cy5 5'- AGA GTT TGA TCC TGG CTC AG -3') (T_m 57.3degC) and
1389R (5'- ACG GGC GGT GTG TAC AAG -3') (T_m 58.2degC)

4min @ 94degC
25 x 1min @ 94degC
1min @ 59degC
1min @ 72degC
1min @ 94degC
1min @ 59degC
5min @ 72degC
Infinity @ 4degC

PCR product purification

Transfer all PCR product (~25ul) to Millipore plate (purple)
Add 75ul of 1 x TE to each sample. Ensure no drops on side of well. Cover top of wells with parafilm
Vacuum on vacuum manifold at 10mmHg until meniscus at bottom of well (not completely dry).
Repeat wash with TE.

Add 60ul of molecular water. Cover with parafilm.
Resuspend on plate shaker at 800rpm for 15 minutes.

Restriction enzyme digestion

Amount of DNA for RE digest depends on the sample bacteria population, primer, label and enzyme used.

The amounts below are just a guide and a few test samples at different concentrations should be run to find the optimum concentration, where most peaks are between 5-80,000rfu. Above this and peaks may be cut off by going over the detection range. Digests incubated for 5 hours at optimum temperature. Digests performed in Beckman sample plate.

	Per reaction (ul)
Molecular water & DNA (50ng)*	44.5
Restriction enzyme (10,000U/ml)	0.5
Buffer (10x)	5.0
Final volume	50.0

Ethanol Precipitation

(performed in sample plate)

Add 4ul of glycogen (20mg/ml) (stored at -20degC), 4ul of EDTA (100mM), 4ul of sodium acetate (pH5.2)

Add 120ul of 95% ethanol (stored at -80degC)

Centrifuge for 30mins at 3000 rpm at 4 degC

Empty tube/plate in 1 movement (**do not re-invert**)

Add 200ul 70% ethanol (stored at -20degC)

Centrifuge for 15 mins at 3000 rpm at 4 degC

Empty tube/plate in 1 movement (**do not re-invert**)

Add 200ul 70% ethanol (stored at -20degC)

Centrifuge for 15 mins at 3000 rpm at 4 degC

Empty plate and **maintain upside down**. Place on blue roll and spin upside down for 10seconds at 300rpm

Leave plate to dry in dark if any ethanol remaining.

Preparation of samples

Add 35ul SLS and 0.2ul size standard 600 to each sample (can make master mix of SLS and standard, and transfer 35ul to each sample). If using a plate, add SLS to any remaining wells to make a complete line of 8.

If using plate, cover with rubber cap

Shake on plate shaker for 20mins at 800rpm

If using tubes, transfer samples to sequencing plate

Add 1 drop of mineral oil on each sample/SLS

Check there are no air bubbles in plate- remove by quickly centrifuging or using pipette tip.

Add separation buffer to buffer plate to match sample plate.

Load samples on a Backman CEQ8800 sequencer. The fluorescent tag on the labelled restriction product is detected automatically and the time of detection relates to the band size.

The output generated is then analysed using the **fragment analysis program** on the computer linked to the DNA sequencer.