Protocol S2: XMRV *gag* nested RT-PCR

Supplement to Urisman et al., *PLOS* Pathogens 2006. "Identification of a Novel Gammaretrovirus in Prostate Tumors of Patients Homozygous for R462Q *RNASEL* Variant."
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Take standard PCR precautions. Wear powder free gloves, use filter tips, UV irradiation boxes and separate areas for sample set up and PCR product analysis.

This protocol consists of three parts: one reverse transcription step and two rounds of amplification. Amplification of human GAPDH gene, used as an endogenous control, is carried out during the first PCR round.

Necessary reagents:

Reverse Transcription:
Reverse Transcriptase (e.g. Superscript II, Invitrogen)
12.5 mM dNTPs
0.1 M DTT
10x Reverse transcription buffer
100 ng/ul random hexamer primer (pdN6)

Round 1 PCR:
5 U/ul Taq polymerase (e.g. recombinant Taq, Invitrogen)
10x Taq buffer
12.5 mM dNTPs
50 mM MgCl2
20 pmol/ul GAG-O-F primer (5'-CGCGTCTGATTTGTTTTGTT)
20 pmol/ul GAG-O-R primer (5'-CCGCCTCTTCTTCATTGTTC)
20 pmol/ul hGAPDH-66F primer (5'-GAAGGTGAAGGTCGGAGTC)
20 pmol/ul hGAPDH-291R primer (5'-GAAGATGGTGATGGGATTTC)

Round 2 PCR:
5 U/ul Taq polymerase (e.g. recombinant Taq, Invitrogen)
10x Taq buffer
12.5 mM dNTPs
50 mM MgCl2
20 pmol/ul GAG-I-F primer (5'-TCTCGAGATCATGGGACAGA)
20 pmol/ul GAG-I-R primer (5'-AGAGGGTAAGGGCAGGGTAA)

Reverse Transcription:
Always include a negative control (water) and a positive control (e.g. NIH3T3 total RNA).

Mix 10-50 ng of sample poly-A RNA with 1ul of pdN6 primer in a final volume of 10ul. Heat sample for 5 min at 65 ˚C. Cool at room temperature for 5 min. Add 10 ul of 2x enzyme mix. Incubate for 90 min at 42 ˚C.

2x enzyme mix:

1.0 ul	10x buffer
0.8 ul	12.5 mM dNTPs
3.2 ul	H_2O
2.0 ul	0.1 M DTT
2.0 ul	RT enzyme

Round 1 PCR:
PCR mix 1 (*gag*):

5.0 ul	10x buffer
2.5 ul	50 mM $MgCl_2$
1.0 ul	12.5 mM dNTPs
0.5 ul	GAG-O-F primer
0.5 ul	GAG-O-R primer
38 ul	H_2O
0.5 ul	Taq
2.0 ul	cDNA

PCR mix 2 (*hGAPDH*):
Prepared as mix 1 with hGAPDH 66F/291R primers

Cycles: 2 min 94 ˚C; [30 sec 94 ˚C, 30 sec 58 ˚C, 45 sec 72 ˚C] x 30 cycles; 7 min 72 ˚C.

Round 2 PCR:
PCR mix:

5.0 ul	10x buffer
2.5 ul	50 mM $MgCl_2$
1.0 ul	12.5 mM dNTPs
0.5 ul	GAG-I-F primer
0.5 ul	GAG-I-R primer
38 ul	H_2O
0.5 ul	Taq
2.0 ul	Round 1 DNA

Cycles: 2 min 94 ˚C; [30 sec 94 ˚C, 30 sec 60 ˚C, 30 sec 72 ˚C] x 30 cycles; 7 min 72 ˚C.

Run 5-7 ul from each PCR round on a 1% agarose gel. Round 1 PCR should amplify a fragment of 612 bp (not always detectable by gel), and Round 2 will result in a fragment of 413 bp. The expected size of the *hGAPDH* fragment is 225 bp.