Parameters, Preparation & qPCR for Gallinacin 1 and 2

Pilot study with Dr. Charles Brockus using 26 chicken tissue RNAs: performed Dec. 14th-16th, 2005

1/6/2006 1:15 PM – reworked 1-17-07 and 1-26-07

After Trizol-based total tissue RNA isolations, a mini-qPCR test, and Turbo DNase treatments, the **Test Plate** for this study looked at a dilution series of total RNA in a mixture made from 50 uL (of post-DNAsed, 1:10-diluted) of each of 26 total RNAs. This mixture had a calculated $o.d._{260nM}$ of 0.0401, and was named "**Stock I.**" The 26 normal chicken tissues were from a male and a female chicken; 13 from each chicken. The tissues were: Bone Marrow, Jejunum, Crop, Testes (male, chicken 1) Oviduct (female, chicken 2), Lung, Skin, Spleen, Liver, Kidney, Bursa, Trachea, Conjunctiva and Tongue. After identifying the optimal RNA dilution ranges for each target, fluorogenic real-time qPCR was carried out.

This RNA mixture ("**Stock I**") was first used on a **Test Plate** to ascertain the optimal RNA dilution ranges for each of 3 targets under investigation; one of which is our chosen housekeeping gene, *Gallus gallus* RIBO 18S

The real-time qPCR targets of primary interest to us here are Gallinacin 1 and Gallinacin 2

(Chicken beta-defensins)

December 14-16th, 2005

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Brockus-Harmon-Gallup-Ackermann

The original form of this document showed qPCR sample dilution values that were all off by the same factor (3.846154). This document now reflects the correct values in each case. The qPCR results remain the same.

From working through the files, we found the lowest theoretically acceptable o.d. 260nm at 1:50 to be **0.00596** (a detail of relatively minor importance – but necessary to note)
(our lowest reading was 0.0905 - from the female trachea)

One can still use samples with lower initial 1:50 readings -- but they will more likely be subject to chemical (or rRNA or target template, etc.) inhibition since they cannot be diluted out to at least 1:200 before use in qPCR.

(Only after a 1:200 dilution of sample RNAs [post Turbo-DNase treatment] have we observed that all forms of qPCR inhibition begin to abate; before that, inhibition of some kind is clearly evident; with these chicken samples, this inhibitory threshold lifted at 1:1000 RNA dilutions and up)

DNase treatments were carried out as follows: 70 uL of each RNA isolate was used in 100 uL-Turbo DNase reactions; each of which became 110 uL after addition of the Turbo kit inactivation reagent.

Then, we removed 80 uL of each DNase-treated RNA from each such rxn, and added 720 uL to each of them (this gave us 800 uL of each sample at 1:10); these were our post-DNase 1:10 pre-dilutions of the sample RNAs from which we prepared our subsequent RNA dilutions (**to fit within the optimal ranges for each target; between the 1st two points on each target's standard curve** – these parameters were all based on what the Test Plate revealed to us)

Again: we took 50 uL of each of the 26 DNase-treated, 1:10 pre-diluted samples and mixed them together to create 1300 uL of a "**Stock I**" solution; we then calculated its o.d. 260nm ... and proceeded, using EXCEL file networks …

It is extremely important to think way ahead as to how much **"Stock I"** will be needed to perform the Test Plate, the 4 sample plates, and the NRC plate at the end …

Quite a juggling act~

~THE SEQUENCES~

NCBI Nucleotide search for G1

1: AF033335. Reports Gallus gallus gal...[gi:3617828] Links

```
LOCUS       AF033335                 504 bp    mRNA    linear   VRT 17-SEP-1998
DEFINITION  Gallus gallus gallinacin 1 prepropeptide (GAL1) mRNA, complete cds.
ACCESSION   AF033335
VERSION     AF033335.1  GI:3617828
KEYWORDS    .
SOURCE      Gallus gallus (chicken)
  ORGANISM  Gallus gallus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Archosauria; Aves; Neognathae; Galliformes; Phasianidae;
            Phasianinae; Gallus.
REFERENCE   1  (bases 1 to 504)
  AUTHORS   Brockus,C.W., Jackwood,M.W. and Harmon,B.G.
  TITLE     Characterization of beta-defensin prepropeptide mRNA from chicken
            and turkey bone marrow
  JOURNAL   Anim. Genet. 29 (4), 283-289 (1998)
   PUBMED   9745666
REFERENCE   2  (bases 1 to 504)
  AUTHORS   Brockus,C.W., Harmon,B.G. and Jackwood,M.W.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-NOV-1997) Veterinary Pathology, University of
            Georgia, College of Veterinary Medicine, Athens, GA 30602, USA
FEATURES             Location/Qualifiers
     source          1..504
                     /organism="Gallus gallus"
                     /mol_type="mRNA"
                     /db_xref="taxon:9031"
                     /cell_type="granulocyte"
     gene            1..504
                     /gene="GAL1"
     CDS             94..291
                     /gene="GAL1"
                     /codon_start=1
                     /product="gallinacin 1 prepropeptide"
                     /protein_id="AAC36051.1"
                     /db_xref="GI:3617829"
                     /translation="MRIVYLLLPFILLLAQGAAGSSQALGRKSDCFRKSGFCAFLKCP
                     SLTLISGKCSRFYLCCKRIWG"
     mat_peptide     169..288
                     /gene="GAL1"
                     /product="gallinacin 1"
                     /function="antimicrobial peptide"
                     /note="beta-defensin"
ORIGIN
        1 ggatgcacgc tgttcttggt ggggttctta cttccttgct gtaccctgag aaaccattgt
       61 cagccctgtg aaaacccggg acagacgtaa accatgcgga tcgtgtacct gctcctcccc
      121 ttcatcctcc tcctggccca gggtgctgca ggatcctccc aggctctagg aaggaagtca
      181 gattgttttc gaaagagtgg cttctgtgca tttctgaagt gcccttccct cactctcatc
      241 agtgggaaat gctcaagatt ttacctctgc tgcaaaagaa tatggggctg aagagccaga
      301 catcccaagc aggacatcac cctggcttct cgcttctgga aacttccccc attgacctct
      361 ccccttccca cctctgcagt ctcccatggt gtgagcgtgg cagtagaagt tggagacatc
      421 ccaccatggg cctgcagttg tttggccagt tgctgctttt ccctgctgaa taaaggtgtg
      481 cagtttagca ttgcaaaaaa aaaa
```

For Chicken (Gallus gallus) Gallinacin 1 real-time primers and probe:
Fwd: 5’- GGAAGGAAGTCAGATTGTTTTCGA
Probe: 5’-6FAM-AGAGTGGCTTCTGTGCATTTCTGAAGTGC-TAMRA
Rev: 5’-GAGCATTTCCCACTGATGAGAGT Designed using primer express V.2.0 3-21-2005 jmg

NCBI Nucleotide search for G2

AF033336. Reports Gallus gallus bet...[gi:3617830] Links

```
LOCUS       AF033336                 423 bp    mRNA    linear   VRT 17-SEP-1998
DEFINITION  Gallus gallus beta-defensin prepropeptide (GAL2) mRNA, complete
            cds.
ACCESSION   AF033336
VERSION     AF033336.1  GI:3617830
KEYWORDS    .
SOURCE      Gallus gallus (chicken)
  ORGANISM  Gallus gallus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Archosauria; Aves; Neognathae; Galliformes; Phasianidae;
            Phasianinae; Gallus.
REFERENCE   1  (bases 1 to 423)
  AUTHORS   Brockus,C.W., Jackwood,M.W. and Harmon,B.G.
  TITLE     Characterization of beta-defensin prepropeptide mRNA from chicken
            and turkey bone marrow
  JOURNAL   Anim. Genet. 29 (4), 283-289 (1998)
   PUBMED   9745666
REFERENCE   2  (bases 1 to 423)
  AUTHORS   Brockus,C.W., Harmon,B.G. and Jackwood,M.W.
  TITLE     Direct Submission
  JOURNAL   Submitted (07-NOV-1997) Veterinary Pathology, University of
            Georgia, College of Veterinary Medicine, Athens, GA 30602, USA
FEATURES             Location/Qualifiers
     source          1..423
                     /organism="Gallus gallus"
                     /mol_type="mRNA"
                     /db_xref="taxon:9031"
     gene            1..423
                     /gene="GAL2"
     CDS             28..222
                     /gene="GAL2"
                     /codon_start=1
                     /product="beta-defensin prepropeptide"
                     /protein_id="AAC36052.1"
                     /db_xref="GI:3617831"
                     /translation="MRILYLLFSLLFLALQVSPGLSSPRRDMLFCKGGSCHFGGCPSH
                     LIKVGSCFGFRSCCKWPWNA"
     mat_peptide     112..219
                     /gene="GAL2"
                     /product="beta-defensin"
                     /function="antimicrobial peptide"
ORIGIN
        1 tatccgcagc tcagcagatc tgcagccatg aggattcttt acctgctttt ctctctcctc
       61 ttcctggcac tccaggtttc tccagggttg tcttcgcccc ggcgggacat gctgttctgt
      121 aaaggagggt cctgccactt tggagggtgt cccagccatc taatcaaagt cggaagctgc
      181 ttcgggttcc gttcctgctg caaatggcct tggaatgcat aaacacttca tgagtccatc
      241 aagagctttg aaaatttctt ccaggcatgt gctttaaatg ctacagcaaa gcctcagcag
      301 caagaagacc cctctcatgt gttaatgcaa tatgttttgt gttgtagagt aaatacaaat
      361 atcttctgca ctgcctttct tcctcttgaa taaattgtca ttgcatagca aaaaaaaaaa
      421 aaa
```

For Chicken (Gallus gallus) Gallinacin 2 real-time primers and probe:
Fwd: 5’-GGAGGGTCCTGCCACTTTG
Probe: 5’-6FAM-AGGGTGTCCCAGCCATCTAATCAA-TAMRA
Rev: 5’-CGGAACCCGAAGCAGCTT
Designed using primer express V.2.0 3-21-2005 jmg

NCBI Nucleotide search for Gallus 18S

THE GREAT HUNT FOR THE CHICKEN 18S rRNA SEQUENCE:

1: K01379. Reports Chicken 18S rRNA ...[gi:174097] Links

```
LOCUS       CHKRRE                    77 bp    rRNA    linear   VRT 20-MAY-1994
DEFINITION  Chicken 18S rRNA 3'-terminal.
ACCESSION   K01379
VERSION     K01379.1  GI:174097
KEYWORDS    18S ribosomal RNA; ribosomal RNA.
SOURCE      Gallus gallus (chicken)
  ORGANISM  Gallus gallus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Archosauria; Aves; Neognathae; Galliformes; Phasianidae;
            Phasianinae; Gallus.
REFERENCE   1  (bases 1 to 77)
  AUTHORS   Azad,A.A. and Deacon,N.J.
  TITLE     The 3'-terminal primary structure of five eukaryotic 18S rRNAs
            determined by the direct chemical method of sequencing. The highly
            conserved sequences include an invariant region complementary to
            eukaryotic 5S rRNA
  JOURNAL   Nucleic Acids Res. 8 (19), 4365-4376 (1980)
   PUBMED   7433112
COMMENT     Original source text: Chicken reticulocyte rRNA.
            [1] found support for the hypothesis that base-paired interaction
            between 5S and 18S rRNA, which are present in the large and small
            ribosomal subunits respectively, may be involved in the reversible
            association of ribosomal subunits during protein synthesis.
FEATURES             Location/Qualifiers
     source          1..77
                     /organism="Gallus gallus"
                     /mol_type="rRNA"
                     /db_xref="taxon:9031"
     rRNA            <1..77
                     /product="18S ribosomal RNA"
     modified_base   57
                     /note="m26a = 2,6-dimethyladenosine"
                     /mod_base=OTHER
     modified_base   58
                     /note="m26a = 2,6-dimethyladenosine"
                     /mod_base=OTHER
ORIGIN
        1 gagtgtgaat tgagtatgta gaggaagtaa aagtcgtaag aaggtttccg taggtgaacc
       61 tgcggaagga gtcatta
```

We found this sequence to slightly agree with the 3’-terminal region of accession #AF173612 …

(CHICKEN 18S rRNA sequence work continued …)

1: M59389. Reports ____ Chicken 18S ribos...[gi:174082] Links

```
LOCUS       CHKRR18S                1779 bp    rRNA    linear   VRT 14-APR-1994
DEFINITION  Chicken 18S ribosomal RNA.
ACCESSION   M59389 M36348
VERSION     M59389.1  GI:174082
KEYWORDS    18S ribosomal RNA.
SOURCE      Gallus gallus (chicken)
  ORGANISM  Gallus gallus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Archosauria; Aves; Neognathae; Galliformes; Phasianidae;
            Phasianinae; Gallus.
REFERENCE   1  (bases 1 to 1779)
  AUTHORS   Hedges,S.B., Moberg,K.D. and Maxson,L.R.
  TITLE     Tetrapod phylogeny inferred from 18S and 28S ribosomal RNA
            sequences and a review of the evidence for amniote relationships
  JOURNAL   Mol. Biol. Evol. 7 (6), 607-633 (1990)
   PUBMED   2283953
COMMENT     Original source text: Chicken liver and egg ribosomal RNA.
FEATURES             Location/Qualifiers
     source          1..1779
                     /organism="Gallus gallus"
                     /mol_type="rRNA"
                     /db_xref="taxon:9031"
                     /tissue_type="liver"
                     /dev_stage="egg"
     gene            1..1779
                     /gene="18S rRNA"
     rRNA            1..1779
                     /gene="18S rRNA"
                     /product="18S ribosomal RNA"
ORIGIN
        1 nnccnggttg atcctgccag tagcannngc tngtctcaaa g attaagcca tgcatgtcta
       61 agtacacacg ggcggtacag tgaaactgcg aatggcnnnn naaatcagtt atggnnnnnn
      121 nnntcgctcc cctcccnnac ttggataact gtngnnnntc tngagctaat ncatgccgac
      181 gagcgccgac ctccgggnac gngnncattt atcagaccaa aaccaacggn cncgcccnnn
      241 ngnnntggnn actctagata acctcgagcc gatcgcagcc ccnntggcgg cgacgaccca
      301 ttnnaatgtc nnccctatca acttncgatg gtactgtctg tgcctaccat ggtgnnnacg
      361 ggtaacgggn aatcagggtt cgatnncgga gagggagcct gagaaacggc tnccacatcc
      421 aagganggca gcaggcgcgc nnattaccca ctcccgaccc gggganntng tnacnaaaaa
      481 taacaataca ggactctttc gaggccctnt nattggaatg agtccacttt aaatnnttta
      541 angaggancc attggagggn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
      601 nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
      661 nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
      721 nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnactttg aaaaaattag
      781 agtgttcaaa gcannnnnnn nnnccgccgg aatnctccag ctaggaataa nggaatagga
      841 ctccngttcn nttttgttng nttcggaaa cggggccatg attaagaggg acggcngggg
      901 gnattcgtat tgtgccgcta gaggtnaaat tcttggaccg gcgcaagacg aactaaagcg
      961 aaagcatttg ccaagaatgt nntcattaat caagaangaa agtcggaggt tcgaagacga
     1021 tcagataccg tngtagttcc gaccataaac gatgccgacn agcgatccgg nngnnnnatt
     1081 cccatnaccc gcnnnnnagc tcccgggaaa cccaagnnnn ngggnnnnnn nnnnnnnnnn
     1141 nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn
     1201 nnnnnnnnnn nnnnnnnaca cgggaaacct nacccgnccn ggacacggac aggatnnaca
     1261 gattgagagc tctttctcga ttccgtgggt nntnnngcat ggcnnnnctt agttggtgga
     1321 gcnntttgtc tngttnattc cgataacgan ngagactctg gcatgctnac tagttacgcg
     1381 acccnngagc ngtcggnntc cannnncgta gagggacaag tggcgttcag ccacccgaga
     1441 ttgagcaata acangtctgt gatgccctta gatgtccgnn nctgcacgcg nnctncactg
     1501 actggctcag cttgtgtcta ccctngccgg naggcgcggg nnacccgttg aaccccattc
     1561 gtgatgggga tcgggnattg caattattcc cnatgaacga ggaattccca gtaagtgcgg
     1621 gtnataagct ngcgttgatt nngtccctnc nnnttgtaca cacngnnnnt tgctactacc
     1681 gattggatgg tttagtgagg tncttggatn ggcnctggng gggtngcnng gcntgnnnnn
     1741 gngnngagga ganggtcgan ntnnactatc tagaggaag
```

IMPORTANT: Here it became apparent to us that the known portion of the rRNA sequence for chicken 18S mimicked its genomic DNA counterpart exactly; there were no introns. This 1994 sequence was updated by the same authors in 2000 (see next page).

(CHICKEN 18S rRNA sequence work continued …)

1: AF173612. Gallus gallus 18S...[gi:7262899]

```
LOCUS       AF173612                1737 bp    DNA     linear   VRT 19-MAR-2000
DEFINITION  Gallus gallus 18S ribosomal RNA gene, complete sequence. ACCESSION   AF173612
VERSION     AF173612.1  GI:7262899
KEYWORDS    .
SOURCE      Gallus gallus (chicken)
  ORGANISM  Gallus gallus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Archosauria; Aves; Neognathae; Galliformes; Phasianidae;
            Phasianinae; Gallus.
REFERENCE   1  (bases 1 to 1737)
  AUTHORS   van Tuinen,M., Sibley,C.G. and Hedges,S.B.
  TITLE     The early history of modern birds inferred from DNA sequences of
            nuclear and mitochondrial ribosomal genes
  JOURNAL   Mol. Biol. Evol. 17 (3), 451-457 (2000)
   PUBMED   10723745
REFERENCE   2  (bases 1 to 1737)
  AUTHORS   van Tuinen,M., Sibley,C.G. and Hedges,S.B.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-JUL-1999) Biology, Pennsylvania State University, 208
            Mueller laboratory, University Park, PA 16802, USA
FEATURES             Location/Qualifiers
     source          1..1737
                     /organism="Gallus gallus"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:9031"
     rRNA            1..1737
                     /product="18S ribosomal RNA"
ORIGIN
        1 attaagccat gcatgtctaa gtacacacgg gcggtacagt gaaactgcga atggctcatt
       61 aaatcagtta tggttccttt ggtcgctccc ctcccgttac ttggataact gtggtaattc
      121 tagagctaat acatgccgac gagcgccgac ctccggggac gcgtgcattt atcagaccaa
      181 aaccaaccgg ggctcgcccg gcggctttgg tgactctaga taacctcgag ccgatcgcac
      241 gcccccgtgg cggcgacgac ccattcgaat gtctgcccta tcaactttcg atggtactgt
      301 ctgtgcctac catggtgacc acgggtaacg gggaatcagg gttcgattcc ggagagggag
      361 cctgagaaac ggctaccaca tccaaggaag gcagcaggcg cgcaaattac ccactcccga
      421 cccggggagg tagtgacgaa aaataacaat acaggactct ttcgaggccc tgtaattgga
      481 atgagtccac tttaaatcct ttaacgagga tccattggag ggcaagtctg gtgccagcag
      541 ccgcggtaat tccagctcca atagcgtata ttaaagttgc tgcagttaaa aagctcgtag
      601 ttggatcttg ggatcgagct ggcggtccgc cgcgaggcga gctaccgcct gtcccagccc
      661 ctgtctctcg gcgccccctc gatgctctta actgagtgtc ccgcggggcc cgaagcgttt
      721 actttgaaaa aattagagtg ttcaaagcag gctggccgcc ggaatactcc agctaggaat
      781 aatggaatag gactccggtt ctattttgtt ggttttcgga aacggggcca tgattaagag
      841 ggacggccgg gggcattcgt attgtgccgc tagaggtgaa attcttggac cggcgcaaga
      901 cgaactaaag cgaaagcatt tgccaagaat gttttcatta atcaagaacg aaagtcggag
      961 gttcgaagac gatcagatac cgtcgtagtt ccgaccataa acgatgccga ctcgcgatcc
     1021 ggcggcgtta ttcccatgac ccgccgggca gctcccggga aacccaagtc tttgggttcc
     1081 ggggggagta tggttgcaaa gctgaaactt aaaggaattg acggaagggc accaccagga
     1141 gtggagcctg cggcttaatt tgactcaaca cgggaaacct cacccggccc ggacacggac
     1201 aggattgaca gattgagagc tctttctcga ttccgtgggt ggtggtgcat ggccgttctt
     1261 agttggtgga gcgatttgtc tggttaattc cgataacgaa cgagactctg gcatgctaac
     1321 tagttacgcg acccccgagc ggtcggcgtc caacttctta gagggacaag tggcgttcag
     1381 ccacccgaga ttgagcaata acaggtctgt gatgccctta gatgtccggg gctgcacgcg
     1441 cgctacactg actggctcag cttgtgtcta ccctacgccg gcaggcgcgg gtaacccgtt
     1501 gaaccccatt cgtgatgggg atcggggatt gcaattattc cccatgaacg aggaattccc
     1561 agtaagtgcg ggtcataagc tcgcgttgat taagtccctg ccctttgtac acaccgcccg
     1621 tcgctactac cgattggatg gtttagtgag gtcctcggat cggccccggc ggggtcggcc
     1681 acggccctgc cggagcgtcg agaagacggt cgaacttgac tatctagagg aagtaaa
```

This is the **DNA** sequence that we used to design our Gallus gallus 18S rRNA primers and probe to: accession #AF173612; again, notice, there are no introns indicated, so we had no choice but to use this.

Notice that there are three "c's" in green font here … these are discrepancies we found between accession # M59389 and # AF173612. We took this into account when designing our primers and probes with Primer Express v.2.0: notice then, how we intentionally avoided the ambiguous region for primers and probe design. This is a good example of the kind of precautions primer and probe designers should take. This is not as easy as it looks on many occasions.

For Chicken Ribosomal 18S RNA primers and probe: based on info from accession 3's M59389 and AF173612:
Fwd: 5'-CCATGGTGACCACGGGTAAC
C-Probe: 5'-VIC-CCCTCTCCGGAATCGAACCCTGATT-TAMRA
Rev: 5'-GGATGTGGTAGCCGTTTCTCA **(**Designed using primer express V.2.0 3-21-2005 jmg)

(CHICKEN 18S rRNA sequence work continued …)

OBFUSCATIVE SEQUENCES ABOUND … (BE AWARE OF THIS) …

1: BQ038294. Reports pgn1c.pk008.o8 no...[gi:20383040] Links

```
LOCUS       BQ038294                 353 bp    mRNA    linear   EST 01-MAY-2002
DEFINITION  pgn1c.pk008.o8 normalized chicken lymphoid cDNA library Gallus
            gallus cDNA clone pgn1c.pk008.o8 5' similar to gb|AF375055.1
            |AF375055 Homo sapiens 18S ribosomal RNA gene, partial sequence;
            and UHRF1 pseudogene, mRNA sequence.
ACCESSION   BQ038294
VERSION     BQ038294.2  GI:20383040
KEYWORDS    EST.
SOURCE      Gallus gallus (chicken)
  ORGANISM  Gallus gallus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Archosauria; Aves; Neognathae; Galliformes; Phasianidae;
            Phasianinae; Gallus.
REFERENCE   1  (bases 1 to 353)
  AUTHORS   Morgan,R.W. and Burnside,J.
  TITLE     Chicken lymphoid ESTs
  JOURNAL   Unpublished (2001)
COMMENT     On Mar 27, 2002 this sequence version replaced gi:19771834.
            Contact: Joan Burnside
            Molecular Endocrinology
            University of Delaware
            40 Townsend Hall, Newark, DE 19717, USA
            Tel: 302 831-1345
            Fax: 302-831-3411
            Email: joan@UDel.Edu, www.chickest.udel.edu.
FEATURES             Location/Qualifiers
     source          1..353
                     /organism="Gallus gallus"
                     /mol_type="mRNA"
                     /db_xref="taxon:9031"
                     /clone="pgn1c.pk008.o8"
                     /sex="Male and Female"
                     /tissue_type="thymus, bursa, spleen, PBL, bone marrow"
                     /lab_host="E.coli EMDH10B"
                     /clone_lib="normalized chicken lymphoid cDNA library"
                     /note="Vector: pCMVSPORT 6"
ORIGIN
        1 ttctacctaa aaatgganna taattatttt tgannttttgg aaanntttttg gttattgtat
       61 atgacaccaa aaagnnntgc ttttgtgaat ggattgtctt tttctaacat aaccaaagtt
      121 tgcagtttaa tacctcagtg anncaannnt gtttttaatc acatttgact gcaaacagac
      181 tggaacaaac gtgcggggtt ttgtttttct taactcttac tcttttaaaa ttataatgtt
      241 aatatgttag ggaaaaatgt tttataggct ttttgctgaa tttgttaatt tttgtacaat
      301 gtgcaagttg aagttttcaa aataaatatc ttttcaaaaa aaaaaaaaaa aag
```

(CHICKEN 18S rRNA sequence work continued …)

THIS AVAILABLE SEQUENCE ALREADY APPEARS WITHIN ACCESSION #AF173612

1: D38360. Reports Gallus gallus gen...[gi:556480] Links

```
LOCUS       CHK18SRR48               293 bp    DNA     linear   VRT 15-MAY-1998
DEFINITION  Gallus gallus gene for 18S rRNA, partial sequence.
ACCESSION   D38360 REGION: <1..>293
VERSION     D38360.1  GI:556480
KEYWORDS    18S ribosomal RNA.
SOURCE      Gallus gallus (chicken)
  ORGANISM  Gallus gallus
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Archosauria; Aves; Neognathae; Galliformes; Phasianidae;
            Phasianinae; Gallus.
REFERENCE   1  (bases 1 to 293)
  AUTHORS   Chikuni,K., Minaka,N. and Ikenaga,H.
  TITLE     Molecular phylogeny of some Passeriformes, based on cytochrome b
            sequences
  JOURNAL   J. Yamashina Inst. for Ornithology 28, 1-8 (1995)
REFERENCE   2  (bases 1 to 293)
  AUTHORS   Chikuni,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-SEP-1994) Koichi Chikuni, National Institute of
            Animal Industry; P.O. Box 5, Tsukuba Norin Kenkyudanchi-kyoku,
            Tsukuba, Ibaraki 305, Japan (E-mail:chikuni@niai.affrc.go.jp,
            Tel:0298-38-8686, Fax:0298-38-8683)
FEATURES             Location/Qualifiers
     source          1..293
                     /organism="Gallus gallus"
                     /mol_type="genomic DNA"
                     /db_xref="taxon:9031"
                     /tissue_type="muscle"
     rRNA            <1..>293
                     /product="18S ribosomal RNA"
ORIGIN
        1 atggttcctt tggtcgctcc cctcccgtta cttggataac tgtggtaatt ctagagctaa
       61 tacatgccga cgagcgccga cctccgggga cgcgtgcatt tatcagacca aaaccaaccc
      121 gggctcgccc ggcggctttg gtgactctag ataacctcga gccgatcgca cgcccccgtg
      181 gcggcgacga cccattcgaa tgtctgccct atcaactttc gatggtactg tctgtgccta
      241 ccatggtgac cacgggtaac ggggaatcag ggttcgattc cggagaggga gcc
```

A LOOK AT WHERE OUR CHICKEN 18S rRNA SET MIGHT FIT WITHIN THE HUMAN 18S RIBOSOMAL RNA SEQUENCE:

The chicken set:
Fwd: CCATGGTGACCACGGGTAAC
C-Probe: VIC-CCCTCTCCGGAATCGAACCCTGATT-TAMRA
Rev: GGATGTGGTAGCCGTTTCTCA

Human Ribosomal 18S RNA:

1: X03205. Reports Human 18S ribosom...[gi:36162]

Green means that this base differs from chicken sequence. The human region that is analogous to the region we used for the chicken set is highlighted in **yellow** below – (albeit highlighted within the human sequence here). The purple, turquoise blue and green shaded regions represent ABI's primers and probe for human 18S rRNA. The blinking region is the ABI set region (if you are viewing this as a word document). The turquoise-shaded base indicates the potentially problematic overlap of ABI's reverse primer and probe (although, since they used the C-probe format, these two oligos do not anneal to the same strand during PCR annealing and extension phases). They need to use the C-probe format since a G would be on the 5' end – quenching the reporter needlessly.

ORIGIN
tacctggttgatcctgccagtagcatatgcttgtctcaaagattaagccatgcatgtctaagtacgcacggccggtacagtgaaactgcgaatgg
ctcattaaatcagttatggttcctttggtcgctcgctcctctcccacttggataactgtggtaattctagagctaatacatgccgacgggcgctg
accccctcgcgggggggatgcgtgcatttatcagatcaaaaccaacccggtcagcccctctccggccccggccgggggcgggcgccggcggct
ttggtgactctagataacctcgggccgatcgcacgcccccgtggcggcgacgacccattcgaacgtctgccctatcaactttcgatggtagtcg
ccgtgccta**ccatggtgaccacgggtgac**gggg**aatcagggttcgattccggagaggg**agcc**tgagaaacggctaccacatcc**aaggaaggcagc
aggcgcgcaaattacccactcccgacccggggaggtagtgacgaaaaataacaatacaggactctttcgaggccctgtaattggaatgagtccac
tttaaatcctttaacgaggatccattggagggcaagtctggtgccagcagccgcggtaattccagctccaatagcgtatattaaagttgctgcag
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cgctagaggtgaaattcttggaccggcgcaagacggaccagagcgaaagcatttgccaagaatgttttcattaatcaagaacgaaagtcggaggt
tcgaagacgatcagataccgtcgtagttccgaccataaacgatgccgaccggcgatgcggcggcgttattcccatgacccgccgggcagcttccg
ggaaaccaaagtctttgggttccgggggagtatggttgcaaagctgaaacttaaaggaattgacggaagggcaccaccaggagtggagcctgcg
gcttaatttgactcaacacgggaaacctcacccggcccggacacggacaggattgacagattgatagctctttctcgattccgtgggtggtggtg
catggccgttcttagttggtggagcgatttgtctggttaattccgataacgaacgagactctggcatgctaactagttacgcgacccccgagcgg
tcggcgtcccccaacttcttagagggacaagtggcgttcagccacccgagattgagcaataacaggtctgtgatgcccttagatgtccggggctg
cacgcgcgctacactgactggctcagcgtgtgcctaccctacgccggcaggcgcgggtaacccgttgaaccccattcgtgatggggatcggggat
tgcaattattccccatgaacgaggaattcccagtaagtgcgggtcataagcttgcgttgattaagtccctgccctttgtacacaccgcccgtcgc
tactaccgattggatggtttagtgaggccctcggatcggccccgccggggtcggcccacggccctggcggagcgctgagaagacggtcgaacttg
actatctagaggaagtaaaagtcgtaacaaggtttccgtaggtgaacctgcggaaggatcatta

We could use our human set for chicken … (if the primers and probe sequences are correct from ABI)
(But we found that the human set did not work very well in the end)

For Chicken Ribosomal 18S RNA primers and probe: based directly on accession # AF173612:
Fwd: 5'-CCATGGTGACCACGGGTAAC
C-Probe: 5'-VIC-CCCTCTCCGGAATCGAACCCTGATT-TAMRA
Rev: 5'-GGATGTGGTAGCCGTTTCTCA

The human region (in blinking lights above when viewed as a word document) is identical to the chicken sequence – so we could reasonably use our human RIBO 18S primers and probe for real-time in chicken. But, the chicken set designed here using the chicken 18S rRNA sequence is good – and that's the one we ordered for use in this project …

~PRIMERS & PROBES~

Gallinacin 1

Cntrl o is the reset file macro | Version: Sept 1st, 2005 | G-1 | Use Primer Express to design probe and primers | a

DETAILED PROCEDURE FOR THE GENE AMP 5700 Sequence Detection System

Gallus | Target: G-1 | Control: Ch18S | Primer compositions: just A, C, T, G (not fluors)

Extinction coefficient contributions:

				G-1 Fwd	G-1 Rev	Ch18S for	Ch18S rev	G-1 probe
	A =	15200	$M^{-1}cm^{-1}$	A = 7	6	5	3	5
	C =	7050	$M^{-1}cm^{-1}$	C = 2	5	6	4	5
	G =	12010	$M^{-1}cm^{-1}$	G = 8	6	6	7	9
	T =	8400	$M^{-1}cm^{-1}$	T = 7	6	3	7	10
fluor	6FAM =	20958	$M^{-1}cm^{-1}$	6FAM = 0	0	0	0	1
quench	TAMRA =	31980	$M^{-1}cm^{-1}$	TAMRA = 0	0	0	0	1

Target primer and probe sequences:
- G-1 Fwd 5'-GGAAGGAAGTCAGATTGTTTTCGA
- G-1 Rev 5'-GAGCATTTCCCACTGATGAGAGT
- G-1 probe 5'-6FAM-AGAGTGGCTTCTGTGCATTTCTGAAGTGC-TAM

intron spanned?

Enter [optimized]: fwd ?nM rev ?nM probe ?nM

Do not have to spec. target probes -- just dilute to 10 uM (see below)

0	Samples (for target and reference)	0	Samples (just target)		From Company arrives as:		
Animal used for optimization cDNA Source:		Chicken Marrow	cDNA opt. 1: 10		G-1 probe	100	uM (60 uL)
Optimum FINAL concentrations used for Reference:			StCv SerDil 1: 3		Ch18S for	10	uM (used as)
Ch18S for ?	nM	cDNA/well	3.000 uL	Max cDNA: 8.550 uL	Ch18S rev	10	uM (used as)
Ch18S rev ?	nM	Volume/well:	30 uL		Gallus Ribo 18S VIC Probe:	10	uM (used as)
Ribo 18S VIC Probe: ?	nM	0.0000000000 uL Water check	Order #1				
Replicates?: 3			used for spec.		Animal type:	Chicken	
			1 uL		# of standards:	0	see co

Volumes from company

	original pmol		(After diluted to what should be 100uM) TE added to get "100 uM"	(1 uL + 99 uL TE) Dilution factor	Absorbances @ 260 nm	cuvette path length (cm)	Sum of ext coef contributions	Achieved Concentrations in uM
	80000	G-1 Fwd	800.00 uL	100	0.2608	1	275380	94.71 uM
Date?	80000	G-1 Rev	800.00 uL	100	0.225	1	248910	90.39 uM
60 uL	6000	G-1 probe	0.00 uL	100	0.356278	1	356278	100.00 uM
100 uL	1000	Ch18S for	0.00 uL	100	0.021556	1	215560	10.00 uM
100 uL	1000	Ch18S rev	0.00 uL	100	0.021667	1	216670	10.00 uM
100 uL	1000	s Ribo 18S VIC F	0.00 uL	100	0.023457	1	234567	10.00 uM

ALL (type in "ALL" or "Part" to connote either diluting entire sample for use, or part of the sample for use)

To Make: 0.6 mL of 10 uM sol'ns.

TE for tot. (after -1 uL for spec)		Sample	1X TE	[final uM]	Final vol.	CALCULATED (-1 uL for spec) Left over conc. stocks		MEASURED (actual)	10 uM stocks xtra possible
6767.97uL	G-1 Fwd	63.35 uL	536.65 uL	10	600.00 uL	G-1 Fwd	0.00 uL	0.00 uL	0.00 uL
6423.49uL	G-1 Rev	66.38 uL	533.62 uL	10	600.00 uL	G-1 Rev	0.00 uL	0.00 uL	0.00 uL
540.00uL	G-1 probe	60.00 uL	540.00 uL	10	600.00 uL	G-1 probe	0.00 uL	0.00 uL	0.00 uL

Forward:	6767.97 uL	3383.98 uL (divided by 2)	1691.99 uL (divided by 4)	966.85 uL	(divided by: 7
Reverse:	6423.49 uL	3211.75 uL (divided by 2)	1605.87 uL (divided by 4)	917.64 uL	(divided by: 7

			added already	need to add
1000 uL	# aliquots of forward primer:	7.6	7567.0 uL F Total	6000	767.97
1000 uL	# aliquots of reverse primer:	7.2	7222.5 uL R Total	6000	423.49
200 uL	# aliquots of TaqMan probe:	3.0			

File for tabulating exact primer and probe sequence data, calculating exact molar extinction coefficients for each, and then used to enter spec. 260nm readings of primers at 1:100 dilutions for the purpose of creating stock mixtures of each oligo which are accurately 10 µM in each case. This is the very first file we use each time we receive a new primer or probe shipment.

Gallinacin 2

Cntrl o is the reset file macro Version: Sept 1st, 2005 G-2 Use Primer Express to design probe and primers a

DETAILED PROCEDURE FOR THE GENE AMP 5700 Sequence Detection System

Gallus | Target: G-2 | Control: Ch18S | Primer compositions: just A, C, T, G (not fluors)

Extinction coefficient contributions:

				G-2 Fwd	G-2 Rev	Ch18S for	Ch18S rev	G-2 probe
	A =	15200	$M^{-1}cm^{-1}$	A = 2	5	5	3	7
	C =	7050	$M^{-1}cm^{-1}$	C = 5	6	6	4	7
	G =	12010	$M^{-1}cm^{-1}$	G = 7	5	6	7	5
	T =	8400	$M^{-1}cm^{-1}$	T = 5	2	3	7	5
fluor	6FAM =	20958	$M^{-1}cm^{-1}$	6FAM = 0	0	0	0	1
quench	TAMRA =	31980	$M^{-1}cm^{-1}$	TAMRA = 0	0	0	0	1

Target primer and probe sequences
G-2 Fwd 5'-GGAGGGTCCTGCCACTTTG
G-2 Rev 5'-CGGAACCCGAAGCAGCTT
G-2 probe 5'-6FAM-AGGGTGTCCCAGCCATCTAATCAA-TAMRA

intron spanned?
Enter [optimized]: fwd ?nM rev ?nM probe ?nM
Do not have to spec. target probes -- just dilute to 10 uM (see below)

0 Samples (for target and reference) | 0 Samples (just target)

Animal used for optimization cDNA Source: Chicken Marrow | cDNA opt. 1: 10 | StCv SerDil 1: 3

Optimum FINAL concentrations used for Reference:
Ch18S for ? nM
Ch18S rev ? nM
RIBO 18S VIC Probe: ? nM

cDNA/well 3.000 uL Max cDNA: 8.550 uL
Volume/well: 30 uL
0.0000000000 uL Water check
Order #1 used for spec. 1 uL

From Company arrives as:

G-2 probe	100	uM (60 uL)
Ch18S for	10	uM (used as)
Ch18S rev	10	uM (used as)
Gallus RIBO 18S VIC Probe:	10	uM (used as)

Replicates?: 3 Animal type: Chicken

Volumes from company (After diluted to what should be 100uM) # of standards: 0 see co

	original pmol		TE added to get "100 uM"	(1 uL + 99 uL TE) Dilution factor	Absorbances @ 260 nm	cuvette path length (cm)	Sum of ext coef contributions	Achieved Concentrations in uM
	80000	G-2 Fwd	800.00 uL	100	0.1691	1	191720	88.20 uM
Date?	80000	G-2 Rev	800.00 uL	100	0.1658	1	195150	84.96 uM
60 uL	6000	G-2 probe	0.00 uL	100	0.310738	1	310738	100.00 uM
100 uL	1000	Ch18S for	0.00 uL	100	0.021556	1	215560	10.00 uM
100 uL	1000	Ch18S rev	0.00 uL	100	0.021667	1	216670	10.00 uM
100 uL	1000	RIBO 18S VIC I	0.00 uL	100	0.023457	1	234567	10.00 uM

ALL (type in "ALL" or "Part" to connote either diluting entire sample for use, or part of the sample for use)

To Make: 0.6 mL of 10 uM sol'ns.

TE for tot. (after -1 uL for spec)	Sample	1X TE	[final uM]	Final vol.	CALCULATED (-1 uL for spec) Left over conc. stocks		MEASURED (actual)	10 uM stocks xtra possible
6248.30uL	G-2 Fwd	68.03 uL	531.97 uL	10	600.00 uL	G-2 Fwd 0.00 uL	0.00 uL	0.00 uL
5989.33uL	G-2 Rev	70.62 uL	529.38 uL	10	600.00 uL	G-2 Rev 0.00 uL	0.00 uL	0.00 uL
540.00uL	G-2 probe	60.00 uL	540.00 uL	10	600.00 uL	G-2 probe 0.00 uL	0.00 uL	0.00 uL

Forward: 6248.30 uL	3124.15 uL (divided by 2)	1562.08 uL (divided by 4)	892.61 uL	(divided by: 7
Reverse: 5989.33 uL	2994.66 uL (divided by 2)	1497.33 uL (divided by 4)	855.62 uL	(divided by: 7

			added already	need to add
1000 uL # aliquots of forward primer:	7.0	7047.3 uL F Total	6000	248.30
1000 uL # aliquots of reverse primer:	6.8	6788.3 uL R Total	5989.33	0.00
200 uL # aliquots of TaqMan probe:	3.0			

File for tabulating exact primer and probe sequence data, calculating exact molar extinction coefficients for each, and then used to enter spec. 260nm readings of primers at 1:100 dilutions for the purpose of creating stock mixtures of each oligo which are accurately 10 µM in each case. This is the very first file we use each time we receive a new primer or probe shipment.

Gallus gallus 18S rRNA

Cntrl o is the reset file macro Version: Sept. 1st, 2005 Ch18S Use Primer Express to design probe and primers a

DETAILED PROCEDURE FOR THE GENE AMP 5700 Sequence Detection System

Gallus | Target: Ch18S | Control: | Primer compositions: just A, C, T, G (not fluors)

Extinction coefficient contributions:

				Ch18S Fwd	Ch18S Rev			Ch18S probe
A =	15200	$M^{-1}cm^{-1}$	A =	5	3			5
C =	7050	$M^{-1}cm^{-1}$	C =	6	4			10
G =	12010	$M^{-1}cm^{-1}$	G =	6	7			4
T =	8400	$M^{-1}cm^{-1}$	T =	3	7			6
fluor 6FAM =	20958	$M^{-1}cm^{-1}$	6FAM =	0	0	0	0	1
quench TAMRA =	31980	$M^{-1}cm^{-1}$	TAMRA =	0	0	0	0	1

Target primer and probe sequences
Ch18S Fwd 5'-CCATGGTGACCACGGGTAAC
Ch18S Rev 5'-GGATGTGGTAGCCGTTTCTCA
Ch18S probe 5'-VIC-CCCTCTCCGGAATCGAACCCTGATT-TAMRA

intron spanned?
Enter [optimized]: fwd ?nM rev ?nM probe ?nM
Do not have to spec. target probes -- just dilute to 10 uM (see below)

0	Samples (for target and reference)	0	Samples (just target)		From Company arrives as:		
Animal used for optimization cDNA Source:		Chicken Marrow	cDNA opt. 1: 10		Ch18S probe	100	uM (60 uL)
Optimum FINAL concentrations used for Reference:			StCv SerDil 1: 3		0	10	uM (used as)
0 ?	nM	cDNA/well	3.000 uL	Max cDNA: 8.550 uL	0	10	uM (used as)
0 ?	nM	Volume/well:	30 uL	Gallus Ribo 18S VIC Probe:		10	uM (used as)
Ribo 18S VIC Probe: ?	nM	0.0000000000 uL Water check	Order #1				
Replicates?: 3			used for spec.		Animal type:	Chicken	
			1 uL		# of standards:	0	see co

Volumes from company

	original pmol		(After diluted to what should be 100uM) TE added to get "100 uM"	(1 uL + 99 uL TE) Dilution factor	Absorbances @ 260 nm	cuvette path length (cm)	Sum of ext coef contributions	Achieved Concentrations in uM
	80000	Ch18S Fwd	800.00 uL	100	0.1941	1	215560	90.04 uM
Date?	80000	Ch18S Rev	800.00 uL	100	0.1976	1	216670	91.20 uM
60 uL	6000	Ch18S probe	0.00 uL	100	0.297878	1	297878	100.00 uM
100 uL	1000	0	0.00 uL	100	0.000000	1	0	#DIV/0! uM
100 uL	1000	0	0.00 uL	100	0.000000	1	0	#DIV/0! uM
100 uL	1000	s Ribo 18S VIC I	0.00 uL	100	0.023457	1	234567	10.00 uM

ALL (type in "ALL" or "Part" to connote either diluting entire sample for use, or part of the sample for use)

To Make: 0.6 mL of 10 uM sol'ns.

TE for tot. (after -1 uL for spec)	Sample	1X TE	[final uM]	Final vol.	CALCULATED (-1 uL for spec) Left over conc. stocks		MEASURED (actual)	10 uM stocks xtra possible
6395.56uL Ch18S Fwd	66.63 uL	533.37 uL	10	600.00 uL	Ch18S Fwd	0.00 uL	0.00 uL	0.00 uL
6487.77uL Ch18S Rev	65.79 uL	534.21 uL	10	600.00 uL	Ch18S Rev	0.00 uL	0.00 uL	0.00 uL
540.00uL Ch18S probe	60.00 uL	540.00 uL	10	600.00 uL	Ch18S probe	0.00 uL	0.00 uL	0.00 uL

Forward: 6395.56 uL	3197.78 uL (divided by 2)	1598.89 uL (divided by 4)	913.65 uL	(divided by: 7	
Reverse: 6487.77 uL	3243.88 uL (divided by 2)	1621.94 uL (divided by 4)	926.82 uL	(divided by: 7	

			added already	need to add
1000 uL # aliquots of forward primer:	7.2	7194.6 uL F Total	6000	395.56
1000 uL # aliquots of reverse primer:	7.3	7286.8 uL R Total	6000	487.77
200 uL # aliquots of TaqMan probe:	3.0			

File for tabulating exact primer and probe sequence data, calculating exact molar extinction coefficients for each, and then used to enter spec. 260nm readings of primers at 1:100 dilutions for the purpose of creating stock mixtures of each oligo which are accurately 10 µM in each case. This is the very first file we use each time we receive a new primer or probe shipment.

Procedure for Primer and Probe dilution

v.8-5-04 Gallup-Grubor, updated 12-12-05 jmg

1.) To each **primer** (which all come as dry lyophilates), **add an amount of nuclease-free TE pH 8.0 which equals the number of pmoles you ordered divded by 100 (e.g. if you ordered 10,000 pmoles, add 100 µL of TE, if you ordered 30,000 pmoles, add 300 uL TE and so on; Enter pmoles for Forward in Cell B24 and pmoles of Reverse in cell B25 in the attached RT-PCR 5700 Diamond 2005.xls file). Always adjust your initial addition of TE pH 8.0 according to how many pmoles you have received.** This now, should give you **approximate** concentrations of each primer of ~**100 µM**. However, this needs to be confirmed via spectrophotometer (so, first warm the spec. UV bulb up for 30 minutes before taking measurements).

2.) **Mix and vortex** each to be sure the oligo pellets/residue in each case have been totally dissolved into the TE pH 8.0 you just added

3.) **Spin** each down in a microfuge briefly

4.) Take **1 µL** of each and add it to **99 µL of TE** pH 8.0 (for a 1:100 dilution), **mix** those solutions well and **spin** down briefly

5.) **Measure** each 1:100 solution in a spectrophotometer at **260 nm** absorbance. Then, in cells **F24** (for forward primer spec. reading) and **F25** (for reverse primer spec. Abs reading) of the file: **RT-PCR 5700 Diamond 2004.xls** enter your spec A_{260nm} readings of your 1:100 dilutions of your primers.

6.) This file uses your Abs **260 nm** values to calculate the **actual concentration** of each primer solution based on the sequence-specific molar extinction coefficients of each oligonucleotide as obtained from the extinction coefficients specific for each base , and the classic nearest neighbor math

7.) **Dilute each primer** solution to **10 µM** according to what you have calculated from your spec. readings for each **(e.g. In the RT-PCR 5700 Diamond 2005.xls file, add the additional µL amount of TE pH 8.0 shown in cell C37 to your forward primer, and add the additional µL amount of TE pH 8.0 shown in cell C38 to your reverse primer).**

8.) To each **probe solution**, you can simply **add 540 µL** of **TE** pH 8.0 and you will have already achieved **10 µM** probe solutions since all probes come to us as 60 µL, 100 µM solutions (and fairly accurately). We have to physically ascertain the amounts of each primer lyophilate since the company even says that they may vary from the "10,000 picomole" mark by 200 or 300 pmoles on most occasions. The only probe that should not be diluted to 10 µM is our probe for **human RIBO 18S** -- it should be diluted to **40 µM** instead (add **90 µL** to the 60 µL, 100 µM solution they send us for that. The 90 µL added will bring the probe to 40 µM (instead of buying the expensive ABI control reagents RIBO 18S kit for this, we order this set as "Nunc39" to avoid getting overcharged by ABI for something that should be public domain).

9.) Once dilutions have been made, aliquot out as desired into nuclease-free 1.5 mL or 200 µL tubes, and store at **-20°C** until use.

~RNA ISOLATION & DNAse TREATMENT~

NOTE:

ALL CHICKEN TISSUES WERE SHIPPED TO US BY DR. HARMON PRESERVED IN RNA*later*™ Catalog No. 76154 (Qiagen)

TRIZOL RNA ISOLATION FROM 0.1g Tissue; Modified Microfuge Method

In a 50 mL nuclease-free tube: 0.1 g tissue + 1 mL Trizol; (for this study, an exact equivalent of this "1.1 mL" was prepared in the form of a slurry as noted in the accompanying EXCEL file to this isolation procedure. Each slurry was made from an entire vial of tissue sample homogenized in 3 mL of Trizol {to create a "wholly representative" sample in each case}, then, using weight per volume calculations in EXCEL during tissue weighing, either Trizol was removed from each prospective tube (before tissue addition) or a portion of each resulting 3 mL + tissue slurry was further diluted by Trizol to obtain a 0.0909 g tissue/mL slurry in each case (0.0833 g/mL Trizol in some cases here) – which was then identical to the amount of tissue per mL we normally extract using Trizol. We stay within these parameters so as not to supercede the RNA extraction capabilities of Trizol itself in accordance with manufacturer recommendations). Samples were then processed as usual: Homogenize 30 sec. Let sit for 5 min. Add 0.2 mL Chloroform, shake vigorously for 15 sec. Let sit for 2-3 min. at room temp. Microfuge @ 4°C for 10 minutes. Transfer (top) aqueous layers to a new 1.5 mL tube (when gathering these water layers, avoid the white middle interface entirely! – this is protein and some genomic DNA). Add 0.5 mL isopropanol, mix, let stand for 10 min. at room temp. Microfuge for 10 minutes @ 4°C. Dump isopropanol (should see nice pellets by now), (all microfuge procedures in this entire procedure are performed at 12,000 x g and 4°C)

We washed each pellet 2X with 1 mL pre-cooled -20C 75% Ethanol: add 1 mL -20°C ethanol, invert tubes to mix, spin at 12,000 x g for 10 minutes (pellets stick well to the bottom as you pour off this first 75% EtOH wash). Vortex with the final 1 mL 75% EtOH wash (dislodge pellet during vortexing here so it is swirling in solution so the 25% water in the ethanol solution can wash away any left over GIT salts from underneath the pellet while the ethanol keeps the RNA precipitated), microfuge 10 minutes (top speed), dump off final 75% EtOH wash. Air dry pellets for 20 minutes to an hour. (Don't over dry – hard to redissolve.)

We resolubilized RNA pellets in a total of 150 uL Ambion nuclease-free water with 0.1 mM EDTA, pH 6.75. (Can also use HPLC-grade water with 0.1 mM EDTA). These solutions are preferably adjusted to pHs <pH 7.0 to prevent divalent cation-dependent and pH-dependent base hydrolysis of RNA). Each sample was then warmed to 65°C for 5 minutes to ensure their total resolubilization.

260nm and 280nm absorbances of a 1:50 dilution of each RNA isolate were measured in a spec. (10 uL RNA sample + 490 uL of the same diluting buffer used for RNA sample solutions above); and spec. was zeroed with the same buffer as well.

RNA absorbances readings of 0.1 and above are desirable. ~0.045 is the lowest acceptable o.d. 260nm at 1:50 dilution … in most practical cases … jmg 8-23-05 11:18 am

SAMPLE OVERVIEW AND PROJECTED USAGE FOR STOCK I CREATION

Sample	ID	Normal
1	Bone Marrow 1	Control
2	Jejunum 1	Control
3	Crop 1	Control
4	Testes 1	Control
5	Lung 1	Control
6	Skin 1	Control
7	Spleen 1	Control
8	Liver 1	Control
9	Kidney 1	Control
10	Bursa 1	Control
11	Trachea 1	Control
12	Conjunctiva 1	Control
13	Tongue 1	Control
14	Bone Marrow 2	Control
15	Jejunum 2	Control
16	Crop 2	Control
17	Oviduct 2	Control
18	Lung 2	Control
19	Skin 2	Control
20	Spleen 2	Control
21	Liver 2	Control
22	Kidney 2	Control
23	Bursa 2	Control
24	Trachea 2	Control
25	Conjunctiva 2	Control
26	Tongue 2	Control

Chicken 1 male

Chicken 2 female

A mixture of these 26 (post-DNased and diluted 1:10) is called "Stock I" and serves as template for cDNA used in primer-probe optimizations, and as the RNA template used to create the standards and inter-plate calibrator RNAs for the Test plate and 5 subsequent sample plates (4 target plates and 1 NRC plate). From each of these 26 Trizol-isolated RNAs, we mixed 50 uL of each (to get 1300 uL) with a calculated 0.0401 collective o.d. 260nm as a solution. It was stored at 4C before use ...

The calculations used to ensure that each Trizol-tissue slurry had similar gram of tissue/mL Trizol values preceding RNA extractions

(This is only a partial view of a much more extensive file)

Trizol RNA isolation Parameters for Chuck's 26 chicken tissues

Chuck's 26 tissues

1	Tissue	0.092	grams (enter value)		cancel
		1	mL Trizol	1.988	
		0.084249	g/mL Tissue	1988	uL removed
		0.9X	usual	close enough	
	used equivalent:	1.186957	mL of slurry	error adj:	0.012
	add:	-0.086957	mL more Trizol		
	to get:	1.1	mL of slurry		
	that is:	0.032106	g/mL Tissue	0.083333	g/mL tissue

3	mL Trizol to each tissue in 50 mL tube		
	Usual isolation parameters		
	Tissue	0.1	grams
		1	mL Trizol
		0.090909	g/mL Tissue
	used:	1.1	mL of slurry
0.1 mg tissue: 150 uL			
0.3 mg tissue: 500 uL		150	uL Final RNA volume
(Ambion nuclease-free H_2O + 0.1mM EDTA, pH 6.75)			

2	Tissue	0.252	grams		cancel
		2.81	mL Trizol	0.228	
		0.082299	g/mL Tissue	228	uL removed
		0.9X	usual	only slight woops	
	used equivalent:	1.22	mL of slurry	error adj:	-0.03800001
	add:	-0.12	mL more Trizol		
	to get:	1.10	mL of slurry		
	that is:	0.090909	g/mL Tissue	0.083333	g/mL tissue

Trizol needed: Estimate	
78	
26	
104	mL

3	Tissue	0.251	grams		cancel
		2.761	mL Trizol	0.239	
		0.083333	g/mL Tissue	239	uL removed
		0.9X	usual		
	used equivalent:	1.20	mL of slurry		
	add:	-0.10	mL more Trizol		
	to get:	1.10	mL of slurry		
	that is:	0.090909	g/mL Tissue	0.083333	g/mL tissue

4	Tissue	0.186	grams		cancel
		2.046	mL Trizol	0.954	
		0.083333	g/mL Tissue	954	uL removed
		0.9X	usual		
	used equivalent:	1.20	mL of slurry		
	add:	-0.10	mL more Trizol		
	to get:	1.10	mL of slurry		
	that is:	0.090909	g/mL Tissue	0.083333	g/mL tissue

5	Tissue	0.471	grams	x	cancel
		3	mL Trizol	0	
		0.135696	g/mL Tissue	-2181	uL
		1.5X	usual		
	used equivalent:	0.74	mL of slurry		
	add:	0.36	mL more Trizol		
	to get:	1.10	mL of slurry		
	that is:	0.090909	g/mL Tissue	0.090909	g/mL tissue

The final attained grams of tissue per mL Trizol slurry in each case (some slight miscalculations here were easily corrected for during the final calculations at the end of this experiment).

grams of tissue/mL slurry

Tissue	Into 1.5 mL tubes … uL Slurry to use	uL Trizol xtra added	final g/mL attained	accuracy
1	1100.00 uL	as is	0.0842	92.7%
2	1100.00 uL	as is	0.0823	90.5%
3	1100.00 uL	as is	0.0833	91.7%
4	1100.00 uL	as is	0.0833	91.7%
5	736.94 uL	363.06 uL	0.0909	100.0%
6	1100.00 uL	as is	0.0833	91.7%
7	638.60 uL	461.40 uL	0.0909	100.0%
8	709.76 uL	390.24 uL	0.0909	100.0%
9	977.19 uL	122.81 uL	0.0909	100.0%
10	1009.09 uL	90.91 uL	0.0909	100.0%
11	1100.00 uL	as is	0.0833	91.7%
12	1100.00 uL	as is	0.0833	91.7%
13	1100.00 uL	as is	0.0833	91.7%
14	1100.00 uL	as is	0.0833	91.7%
15	1028.79 uL	71.21 uL	0.0909	100.0%
16	897.87 uL	202.13 uL	0.0909	100.0%
17	1100.00 uL	as is	0.0833	91.7%
18	1100.00 uL	as is	0.0833	91.7%
19	1100.00 uL	as is	0.0833	91.7%
20	607.61 uL	492.39 uL	0.0909	100.0%
21	949.86 uL	150.14 uL	0.0909	100.0%
22	1100.00 uL	as is	0.0833	91.7%
23	1100.00 uL	as is	0.0833	91.7%
24	1100.00 uL	as is	0.0833	91.7%
25	1100.00 uL	as is	0.0833	91.7%
26	1100.00 uL	as is	0.0833	91.7%

1.1 mL (1100 uL) of slurry was subjected to RNA isolation in each case. All 26 samples yielded ample RNA amounts.

The resulting o.d. 260nm and 260nm/280nm RNA purity ratios were as follows:

TABATHA Data Entry File 2005 | RNA was isolated from? Tissue ←(type "Cells" or "Tissue") vol adjust: -350 uL | dil. factor 1: 50 | j.m.g. 12-14-05

DATA ENTRY SHEET: RNA sample I.D.'s, o.d.'s @ 260nm, and 260nm/280nm ratios

150 uL — Dilution factor is 1: 50 — desired o.d.is > 0.03642

Group a	Sample ID	o.d. 260nm	260/280 ratio
1	1	0.2775	1.6080
2	2	0.9395	1.7171
3	3	0.2738	1.6347
4	4	0.7482	1.8235
5	5	0.7271	1.6762
6	6	0.6323	1.6755
7	7	0.5825	1.7188
8	8	1.1083	1.9876
9	9	1.3576	1.7251
10	10	0.6544	1.7392

150 uL — Dilution factor is 1: 50 — desired o.d.is > 0.03642

Group b	Sample ID	o.d. 260nm	260/280 ratio
1	11	0.2416	1.7392
2	12	0.4032	1.6374
3	13	0.6569	1.6898
4	14	0.1837	1.6825
5	15	0.6289	1.7487
6	16	0.6033	1.6784
7	17	0.1016	1.6374
8	18	0.6156	1.6738
9	19	0.1160	1.6627
10	20	0.5848	1.6999

150 uL — Dilution factor is 1: 50

Group c	Sample ID	o.d. 260nm	260/280 ratio
1	21	0.6167	1.7131
2	22	1.1139	1.7255
3	23	2.0829	1.6401
4	24	0.0905	1.6399
5	25	0.4179	1.6330
6	26	0.6262	1.7261
7			
8			
9			
10			

(The fact that most of the purity ratios were between 1.6 and 1.74 (instead of nearer to 2.0) indicates that Qiagen RNA Later had some affect on the composition of our final isolates, i.e. it affected what kind of contaminating proteinacious materials or bio-molecules carried over into our final RNA samples as a result of Trizol RNA isolation). These depressed purity ratio values presented no consequence in this study, however.

The thermocycling program for Turbo DNase treatment is 30 minutes at 37C followed by a hold at room temp. This is followed immediately by addition of the Turbo kit's inactivation reagent to each tube – after which each sample is vortexed every 10-15 seconds for 2 minutes and spun for 1.5 min. at 10,000 x g (to pellet the inactivation reagent). The pelleted inactivation reagent is avoided when retrieving RNA from each tube as it can inhibit qPCR rxns by removing enzymes from solution …

RNA volumes available: 140 uL

When having to use fixed volumes of each RNA for DNase treatment reactions:

So your DNase set-ups will each be: 100 uL

adjst all: 1

Made up of:	
10.00 uL	10X DNase Buffer
0.00 uL	nuclease-free Water
20.00 uL	DNase Enzyme (e.g. Turbo DNase)
70.00 uL	RNA isolate
10.00 uL	Inactivation Reagent/Stop solution
Final post DNase volume: 110.0 uL	

Turbo-treated RNA sample should comprise no more than 40% of any final real-time RT-PCR rxn volume … (we use 26%)

A whopping dose of TurboDNAse was delivered to each DNAse-treatment reaction (enough to DNAse treat 200 ug of RNA in each case). Interestingly, the samples which most resisted DNase Treatments were: the male chicken's tongue and the female chicken's lung, crop, jejunum and trachea – tissues high in collagen?

THE CONCENTRATIONS OF EACH ORIGINAL RNA ISOLATE:

1	0.555	ug/uL	BoneM1
2	1.879	ug/uL	Jej1
3	0.5476	ug/uL	Crop1
4	1.4964	ug/uL	Testes1
5	1.4542	ug/uL	Lung1
6	1.2646	ug/uL	Skin1
7	1.165	ug/uL	Spleen1
8	2.2166	ug/uL	Liver1
9	2.7152	ug/uL	Kidny1
10	1.3088	ug/uL	Bursa1
11	0.4832	ug/uL	Trach1
12	0.8064	ug/uL	Conj1
13	1.3138	ug/uL	Tongue1
14	0.3674	ug/uL	BoneM2
15	1.2578	ug/uL	Jej2
16	1.2066	ug/uL	Crop2
17	0.2032	ug/uL	Ovid2
18	1.2312	ug/uL	Lung2
19	0.232	ug/uL	Skin2
20	1.1696	ug/uL	Spleen2
21	1.2334	ug/uL	Liver2
22	2.2278	ug/uL	Kidny2
23	4.1658	ug/uL	Bursa2
24	0.181	ug/uL	Trach2
25	0.8358	ug/uL	Conj2
26	1.2524	ug/uL	Tongue2

Notice that Bursa2 RNA would normally be considered outside the range of Turbo-DNAse's capabilities as we used it here – we added 40 Units DNAse to each 100 uL reaction (enough to DNAse-treat 200 µg of RNA according to Ambion's product literature). The 70 uL used of this RNA sample for DNAse treatment contained about 293 ug RNA. It cleaned up just fine as you can see later on in this document - no problems were encountered.

Based on the o.d. 260nm readings for each sample, the 26 resulting 150 uL-RNA solutions (of which 10 uL of each was used for spec. readings) had the above total RNA concentrations. 70 uL of each was then taken and DNase treated (as shown on the previous page), leaving 70 uL of each left over for storage at 4°C in the event of an emergency … After DNase treatments were inactivated and spun down at 10,000 x g for 1.5 minutes, 80 uL of each DNAsed RNA was taken and added to 720 uL nuclease-free water and vortexed; these now became the post-DNAse 1:10-diluted RNA samples from which all other samples were prepared for this study …

~THE MINI pre-TEST PLATE~

Mini pre-Test qPCR run on non-DNased bone marrow RNA isolate:

During the RNA preparations above (just after resolubilization of samples into 150 uL of water + 0.1mM EDTA pH 6.75 and during the DNase treatments of all samples), we took a small portion of the (non-DNAse-treated) male chicken bone marrow total RNA isiolate (known to be a strong positive control for G1 and G2 from prior studies by Brockus and Harmon) and quickly tested it for target presence by one-step real-time qPCR to see for the very first time if our never-before-tried (nor optimized) primers and probes for chicken G1, G2 and 18S rRNA were indeed going to work at all. In case the chicken 18S rRNA set didn't work, we added human 18S rRNA primers and probes as well (as a potential back-up if the chicken housekeeper set failed for some unforeseen reason; which it didn't).

Print Out (p. 5 of this file)

One-Step Real-Time RT-PCR Set-Up

32 uL prepared/Well	One-Step MM	547.62 uL	Set 1/sample	119.88 uL	xtra ea.made
	RT	27.38 uL	Set 1/MM ea	167.83 uL	
25 uL used/Well	Total MMRT prepared:	575.00 uL	Set 2/sample	0.00 uL	
	Total MMRT needed:	476.28 uL	Set 2/MM ea	0.00 uL	

Well size prepared: 32 uL
RNA added/well: 8.42 uL

Duplicates: 4 samples prepared; 8 wells prepared

Other

98.72 uL extra made

Mini Test Plate
Dec. 14, 2005
Brockus Project

G-1				G-2			
Fwd primer	20.41 uL	split		Fwd primer	20.41 uL	split	
Rev primer	20.41 uL	into		Rev primer	20.41 uL	into	
Probe	2.27 uL	1		Probe	2.27 uL	1	
MMRT:	119.07 uL	47.95 uL	amounts	MMRT:	119.07 uL	47.95 uL	amounts
Water:	5.67 uL	then add	16.85 uL	Water:	5.67 uL	then add	16.85 uL
			RNA to each				RNA to each

Tested on "1:500" -diluted non-DNAsed male chicken bone marrow RNA -- just after it got resolubilized:
I took 1 uL of it and added 129 uL nuc-free water to it for a 1:500 dilution … since a 1:3.846 dilution happens in-well

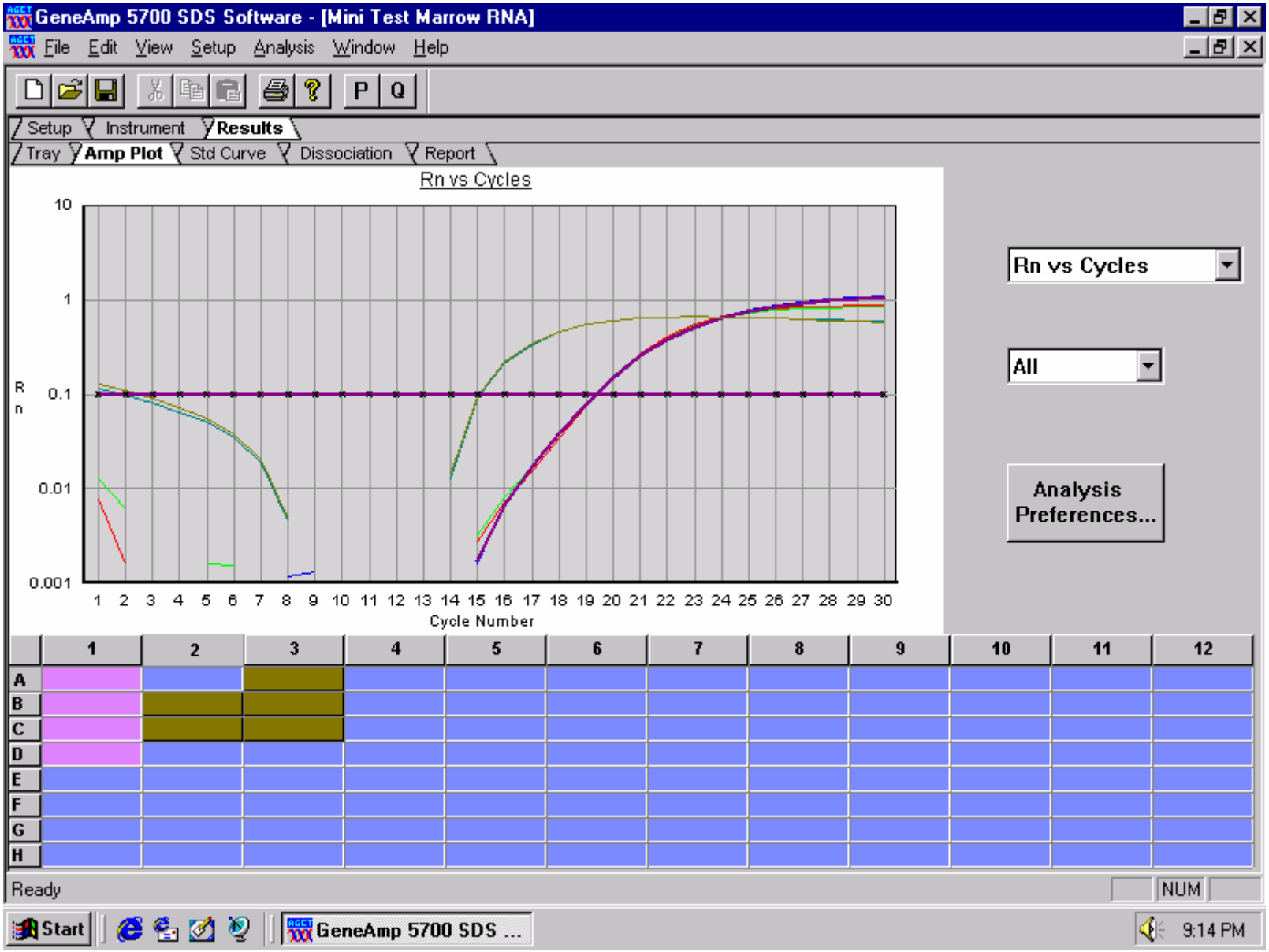
All primers were at 900nM and probes were at 100nM for this plate … (except h18S)
35 cycles were run

Ch18S				RIBO 18S			
Fwd primer	20.41 uL	split		Fwd primer	1.13 uL	split	
Rev primer	20.41 uL	into		Rev primer	1.13 uL	into	
Probe	2.27 uL	1		Probe	1.13 uL	1	
MMRT:	119.07 uL	47.95 uL	amounts	MMRT:	119.07 uL	47.95 uL	amounts
Water:	5.67 uL	then add	16.85 uL	Water:	45.36 uL	then add	16.85 uL
			RNA to each				RNA to each

We also set up singlet NTC wells for each target with the extra Master Mix made in each case …

Note: we took 1 uL (0.555 ug) of the original male chicken bone marrow total RNA isolate, added 129 uL of nuclease-free water to it, and this was used as the RNA source for the Mini pre-Test qPCR Plate set-up shown above. The marrow RNA sample ended up at a 1:500 dilution in well here: 1.11 ng/uL, and this small test showed us that the best had happened; all 3 brand-new primer-probe real-time qPCR sets worked like a charm (see below):

GeneAmp 5700 depiction of the amplifications resultant of the pre-Mini qPCR Test:



G1 and G2 co-amplified in nearly identical regions (Ct = ~19.4), and the chicken 18S rRNA signal amplified (with an expected inhibitory characteristic) at a Ct of ~15 cycles … what a happy sight this was; we could then proceed with complete confidence throughout the remainder of the procedure. Note: human 18S rRNA amplified quite poorly with chicken RNA – (curve not shown here).

~THE TEST PLATE~

THE TEST PLATE SET-UP:

Machine Factors:	2.60E-02	2.00E-02	1.00E-02	5.00E-03	2.00E-03	1.00E-03	2.00E-04	1.00E-04	2.00E-05	2.00E-06	2.00E-07	*12/15/2005*
	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	1:38.46	1:50	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000
B	NTC	1:38.46	1:50	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000
C	NTC	1:38.46	1:50	1:100	1:200	1:500	1:1000	1:5000	1:10000	1:50000	1:500000	1:5000000
D												
E												
F												
G												
H												

G-1

G-2

Ch18S

Tested Concentrations to see where inhibition of any kind lets up for each different target

Initial RNA is already at 1: 10 → **in well will actually be a 1: 38.46**

After DNAse treatment:

samples are diluted 1: 10

And samples, after further dilutions, are then used in-well at a proportion of:

7.80 uL sample

30.00 uL well size

sample fraction is thus: 0.26

Desired final in-well test dilution 1: 50
Desired final in-well test dilution 1: 100
Desired final in-well test dilution 1: 200
Desired final in-well test dilution 1: 500
Desired final in-well test dilution 1: 1000
Desired final in-well test dilution 1: 5000

Desired final in-well test dilution 1: 10000
Desired final in-well test dilution 1: 50000
Desired final in-well test dilution 1: 500000
Desired final in-well test dilution 1: 5000000

Given that our Stock I Solution RNA mixture is calculated to be: 80.2058 ng/uL (comprised of 1:10 RNAs)

This Test Plate dilution series thus represents:

Stock I range tested

20.85351	ng/uL in well
16.04116	ng/uL in well
8.02058	ng/uL in well
4.01029	ng/uL in well
1.604116	ng/uL in well
0.802058	ng/uL in well
0.160412	ng/uL in well
0.080206	ng/uL in well
0.016041	ng/uL in well
0.001604	ng/uL in well
0.00016	ng/uL in well

THE SERIAL DILUTIONS OF STOCK I RNA USED TO CREATE THE SAMPLES FOR THE TEST PLATE:

Stock I

COMPREHESIVE SERIAL DILUTION TABLE

	total made	"reagent"	diluent	to next	(FINAL VOL.)	Achieved Dilutions 1:	Actual Final Dilutions Achieved for Test Plate after used in-well:
A							
B	383.2 uL	294.8 uL	88.4 uL	183.2 uL	200.0 uL	1.3	1: 50
C	366.4 uL	183.2 uL	183.2 uL	166.4 uL	200.0 uL	2.6	1: 100
D	332.9 uL	166.4 uL	166.4 uL	132.9 uL	200.0 uL	5.2	1: 200
E	332.2 uL	132.9 uL	199.3 uL	132.2 uL	200.0 uL	13.0	1: 500
F	264.4 uL	132.2 uL	132.2 uL	64.4 uL	200.0 uL	26.0	1: 1000
G	322.2 uL	64.4 uL	257.8 uL	122.2 uL	200.0 uL	130.0	1: 5000
H	244.4 uL	122.2 uL	122.2 uL	44.4 uL	200.0 uL	260.0	1: 10000
I	222.0 uL	44.4 uL	177.6 uL	22.0 uL	200.0 uL	1300.0	1: 50000
J	220.0 uL	22.0 uL	198.0 uL	20.0 uL	200.0 uL	13000.0	1: 500000
K	200.0 uL	20.0 uL	180.0 uL		200.0 uL	130000.0	1: 5000000

Note: that the starting "reagent" here is the Post-DNased, 1:10 diluted "Stock I" RNA mixture prepared from mixing 50 uL of each of the post-DNAse-treated, 1:10-diluted 26 RNAs in this study (as already described)

THE MASTER MIX SET-UPS FOR THE TEST PLATE:

One-Step Real-Time RT-PCR Set-Up

	One-Step MM	876.19 uL	Set 1/sample	111.00 uL	xtra ea.made
30 uL prepared/Well	RT	43.81 uL	Set 1/MM ea	377.40 uL	Singlets
25 uL used/Well	Total MMRT prepared:	920.00 uL	Set 2/sample	0.00 uL	
	Total MMRT needed:	803.25 uL	Set 2/MM ea	0.00 uL	Other

Well size prepared:	30 uL
RNA added/well:	7.80 uL
36 samples prepared	
36 wells prepared	

116.75 uL extra made

Test PLATE
Dec. 15th, 2005
Brockus Project

G-1

Fwd primer	51.00 uL	split		
Rev primer	51.00 uL	into		
Probe	7.65 uL	12		
MMRT:	267.75 uL	22.20 uL	amounts	
Water:	0.00 uL	then add	7.80 uL	
			RNA to each	

G-2

Fwd primer	51.00 uL	split		
Rev primer	51.00 uL	into		
Probe	7.65 uL	12		
MMRT:	267.75 uL	22.20 uL	amounts	
Water:	0.00 uL	then add	7.80 uL	
			RNA to each	

This Test plate we set up by hand - not by robot

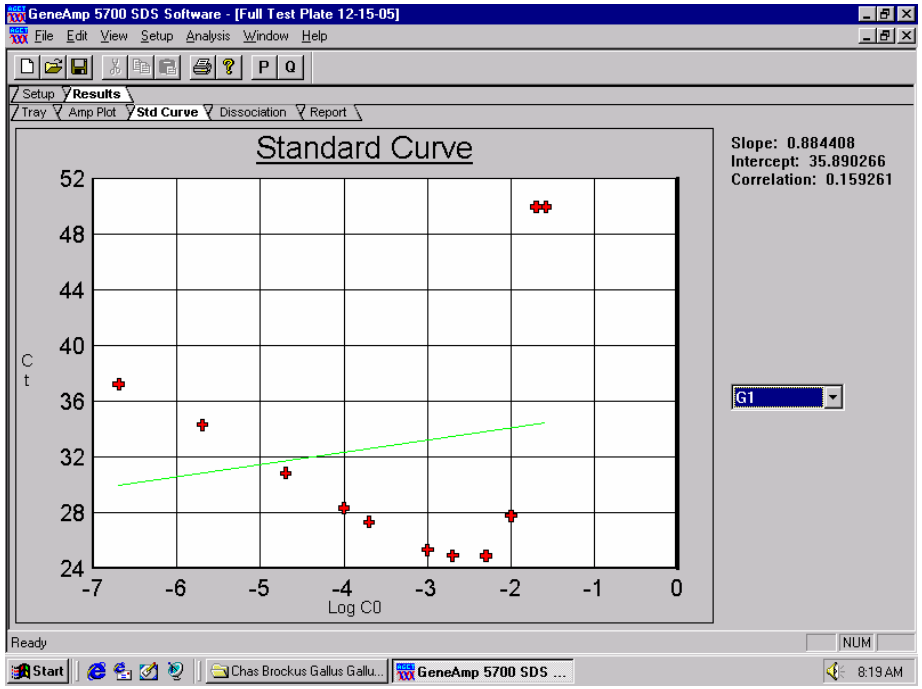
Ch18S

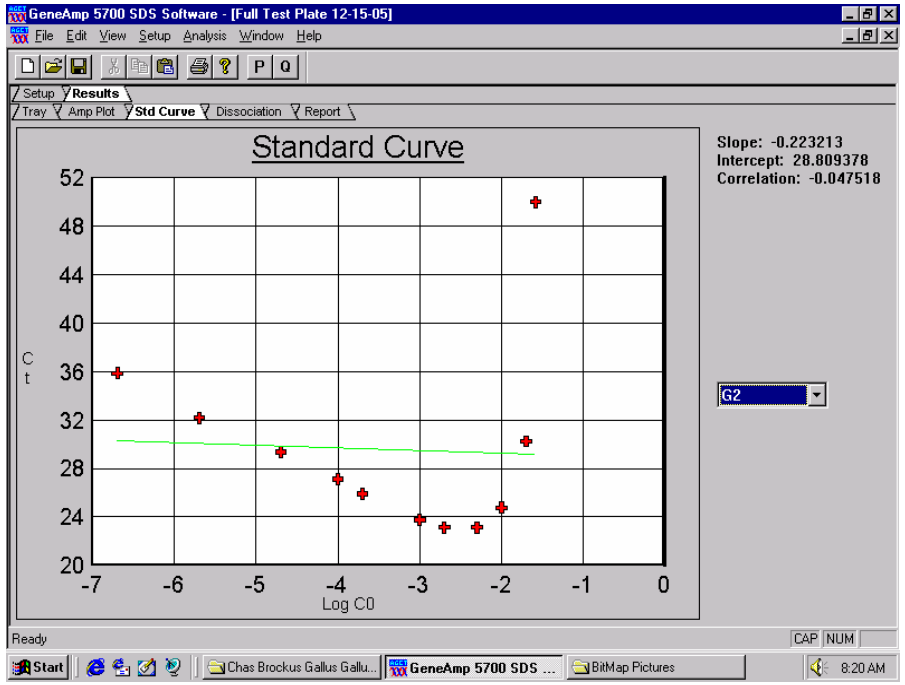
Fwd primer	51.00 uL	split		
Rev primer	51.00 uL	into		
Probe	7.65 uL	12		
MMRT:	267.75 uL	22.20 uL	amounts	
Water:	0.00 uL	then add	7.80 uL	
			RNA to each	

RESULTS FOR G1 AND G2 FROM THE TEST PLATE – SHOWING INHIBITORY AND NON-INHIBITORY DILUTION RANGES:

Well	Type	Name	Primer/Probe	Ct	StdDev Ct	Qty
A1	NTC	G1	G1	50	0	
A2	STND		G1	50	0	2.60E-02
A3	STND		G1	50	0	2.00E-02
A4	STND		G1	27.65	0	1.00E-02
A5	STND		G1	24.94	0	5.00E-03
A6	STND		G1	24.89	0	2.00E-03
A7	STND		G1	25.35	0	1.00E-03
A8	STND		G1	27.4	0	2.00E-04
A9	STND		G1	28.39	0	1.00E-04
A10	STND		G1	30.78	0	2.00E-05
A11	STND		G1	34.24	0	2.00E-06
A12	STND		G1	37.51	0	2.00E-07
B1	NTC	G2	G2	50	0	
B2	STND		G2	50	0	2.60E-02
B3	STND		G2	29.84	0	2.00E-02
B4	STND		G2	24.82	0	1.00E-02
B5	STND		G2	23.27	0	5.00E-03
B6	STND		G2	23.23	0	2.00E-03
B7	STND		G2	23.9	0	1.00E-03
B8	STND		G2	26.02	0	2.00E-04
B9	STND		G2	27.12	0	1.00E-04
B10	STND		G2	29.44	0	2.00E-05
B11	STND		G2	32.67	0	2.00E-06
B12	STND		G2	36.16	0	2.00E-07

Baseline cut-off at Cycle 20; threshold at 0.1

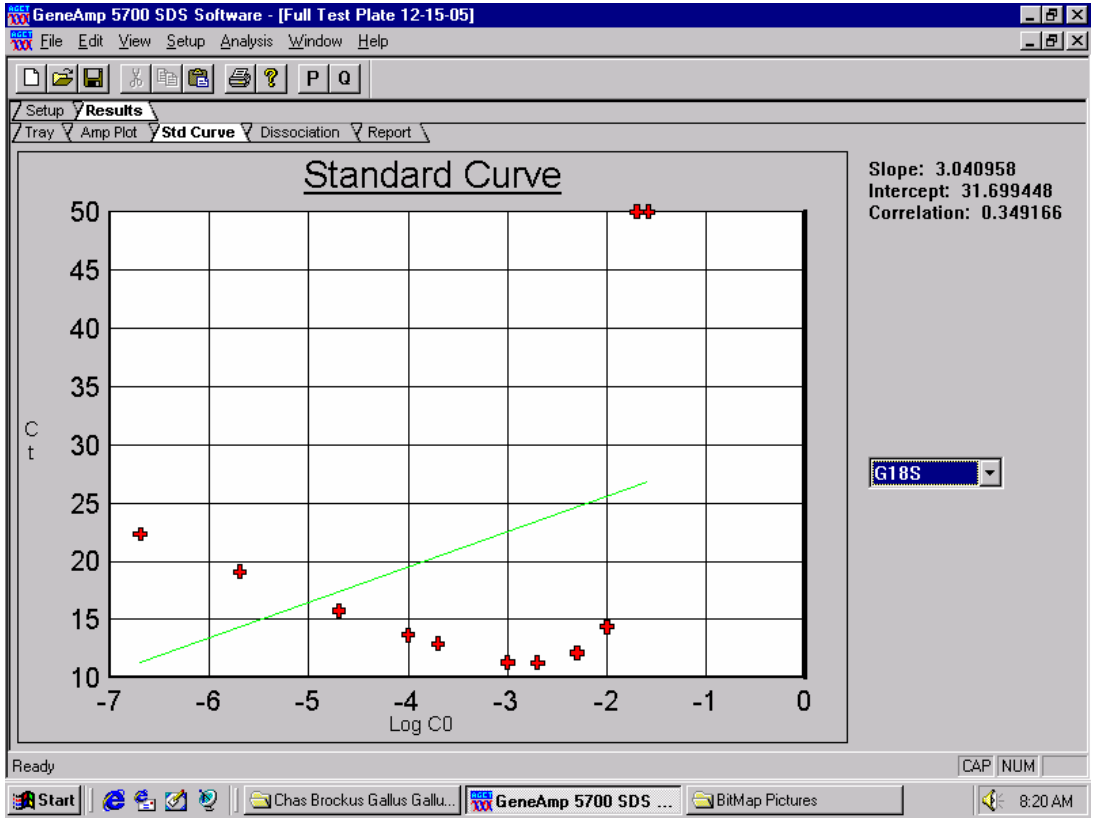




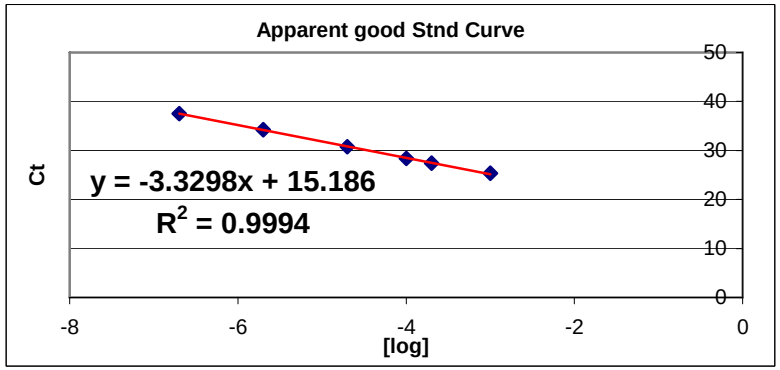
RESULTS FOR Gallus gallus 18S rRNA FROM THE TEST PLATE – SHOWING INHIBITORY AND NON-INHIBITORY DILUTION RANGES:

Well	Type	Name	Primer/Probe	Ct	StdDev Ct	Qty
C1	NTC	G18S	G18S	36.56	0	
C2	STND		G18S	50	0	2.60E-02
C3	STND		G18S	50	0	2.00E-02
C4	STND		G18S	14.11	0	1.00E-02
C5	STND		G18S	11.4	0	5.00E-03
C6	STND		G18S	10.49	0	2.00E-03
C7	STND		G18S	10.77	0	1.00E-03
C8	STND		G18S	12.75	0	2.00E-04
C9	STND		G18S	13.5	0	1.00E-04
C10	STND		G18S	15.56	0	2.00E-05
C11	STND		G18S	19.02	0	2.00E-06
C12	STND		G18S	22.02	0	2.00E-07

Baseline cut-off at Cycle 8; threshold at 0.1



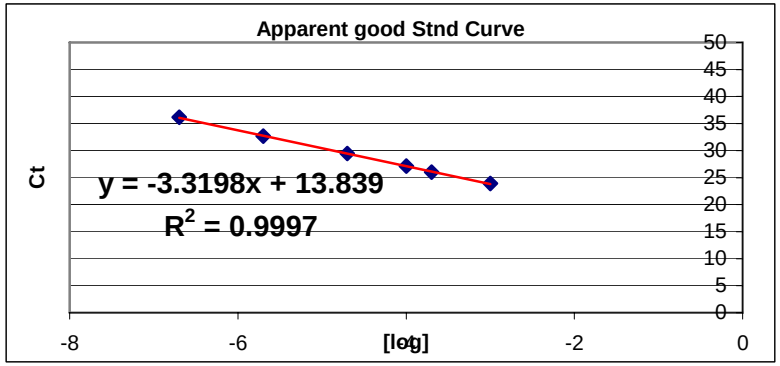
DEPICTION OF A PORTION OF THE FILE WE USE TO HONE IN ON THE OPTIMAL RNA DILUTION RANGES FOR EACH TARGET:



G1

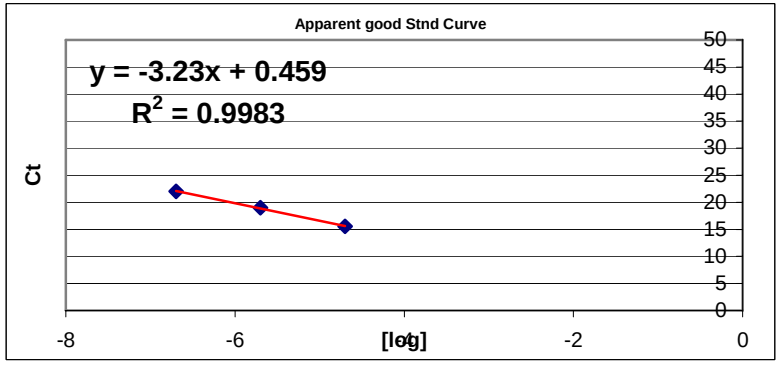
		suggested	Stnd. Curve "in-well"
start useful stnd curve at 1:		1000	1000
serial 1:		10	10000
Efficiency:	99.67%	119.26% (Better E)	100000
Correlation:	-1.000		1000000
b =	15.186		
m =	-3.3298		

Ctrl+Shift+B to run both files



G2

		suggested	Stnd. Curve "in-well"
start useful stnd curve at 1:		1000	1000
serial 1:		10	10000
Efficiency:	100.09%	113.65% (Better E)	100000
Correlation:	-1.000		1000000
b =	13.839		
m =	-3.3198		



chR18S

		suggested	Stnd. Curve "in-well"
start useful stnd curve at 1:		50000	50000
serial 1:		5	250000
Efficiency:	103.98%	115.44% (Better E)	1250000
Correlation:	-0.999		6250000
b =	0.459		
m =	-3.2300		

EXCELLENT RANGES FOR EACH TARGET WERE IDENTIFIED – RANGES WHICH ALSO ALLOWED US TO ATTAIN VIRTUALLY 100% REACTION EFFICIENCIES IN EACH CASE!

MORE PORTIONS OF THE TEST PLATE DATA PROCESSING FILE ABOVE …

Well	Type	Name	Primer/Probe	Paste Cts	Notes:	Qty	Sept. 16th, 2005 to 11-18-06 jmg		
A1	NTC	G1	G1	50		dil factor 1: 1			
A2	UNKN	38.46154	G1	50	ideal Cts:	1	or:	1	
A3	UNKN	50	G1	50	50.38	0.2		0.5	
A4	UNKN	100	G1	27.65	51.00	0.004		0.1	
A5	UNKN	200	G1	24.94	28.65	0.00004		0.05	
A6	UNKN	500	G1	24.89	26.26	1.91E-09			
A7	UNKN	1000	G1	25.35	27.26	1.91E-09			
A8	UNKN	5000	G1	27.4	29.58	0.062684			
A9	UNKN	10000	G1	28.39	30.58	0.531216			
A10	UNKN	50000	G1	30.78	32.91	0.076072			
A11	UNKN	500000	G1	34.24	36.23	0.035344			
A12	UNKN	5000000	G1	37.51	39.55	0.000381			
B1	NTC	G2	G2	50		dil factor 1: 1			
B2	UNKN	38.46154	G2	50	ideal Cts:	1		1	
B3	UNKN	50	G2	29.84	50.38	0.2		0.2	
B4	UNKN	100	G2	24.82	30.84	0.004		0.1	
B5	UNKN	200	G2	23.27	25.82	0.00004		0.02	
B6	UNKN	500	G2	23.23	24.59	8.79E-10			
B7	UNKN	1000	G2	23.9	25.59	0.003834			
B8	UNKN	5000	G2	26.02	27.91	0.172579			
B9	UNKN	10000	G2	27.12	28.91	0.576289			
B10	UNKN	50000	G2	29.44	31.24	0.069467			
B11	UNKN	500000	G2	32.67	34.56	0.030165			
B12	UNKN	5000000	G2	36.16	37.88	0.000448			
C1	NTC	chR18S	chR18S	36.56		dil factor 1: 1			
C2	UNKN	38.46154	chR18S	50	ideal Cts:	1		1	
C3	UNKN	50	chR18S	50	50.38	0.2		0.2	
C4	UNKN	100	chR18S	14.11	51.00	0.004		0.1	
C5	UNKN	200	chR18S	11.4	15.11	0.00004		0.02	
C6	UNKN	500	chR18S	10.49	12.72	3.3E-16			
C7	UNKN	1000	chR18S	10.77	13.72	3.3E-16			
C8	UNKN	5000	chR18S	12.75	16.04	0.028018			
C9	UNKN	10000	chR18S	13.5	17.04	0.711771			
C10	UNKN	50000	chR18S	15.56	19.37	0.094459			
C11	UNKN	500000	chR18S	19.02	22.69	0.048325			
C12	UNKN	5000000	chR18S	22.02	26.01	0.000348			

select which x data to use

For BPO Publication

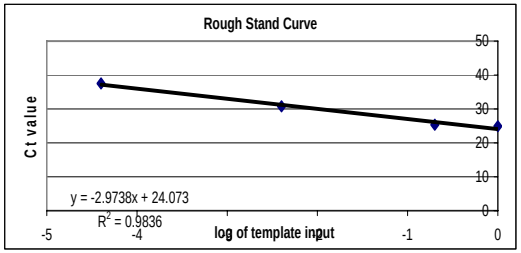
	log of input x	Ct y
adj. values	0	24.94
	-0.69897	25.35
	-2.39794	30.78
	-4.39794	37.51

	log of input x	Ct y
adj. values	0	23.27
	-0.69897	23.9
	-2.39794	29.44
	-4.39794	36.16

	log of input x	Ct y
adj. values	0	11.4
	-0.69897	10.77
	-2.39794	15.56
	-4.39794	22.02

Ideal ΔCt Spread
5.265
0.379
1.000
1.000
1.322
1.000
2.322
1.000
2.322
3.322
3.322

G1 116.91% Efficiency



Correlation: -0.99176

b = 24.07302

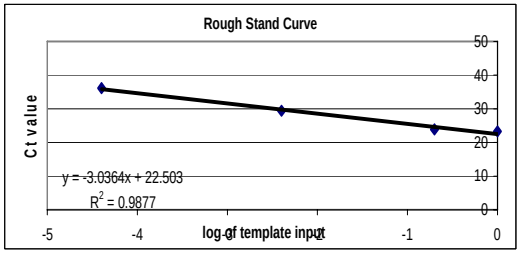
m = -2.973764

NTC here = 50.00

Quantities of Unknowns

0.00000
0.00000
0.06268
0.53122
0.07607
0.03534
0.00038

G2 113.47% Efficiency



Correlation: -0.993831

b = 22.50322

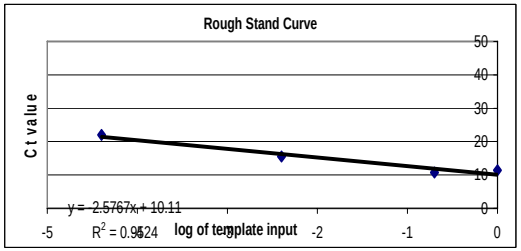
m = -3.03637

NTC here = 50.00

Quantities of Unknowns

0.00000
0.00383
0.17258
0.57629
0.06947
0.03017
0.00045

chR18S 144.40% Efficiency



Correlation: -0.975894

b = 10.10953

m = -2.576688

NTC here = 36.56

Quantities of Unknowns

0.00000
0.00000
0.02802
0.71177
0.09446
0.04833
0.00035

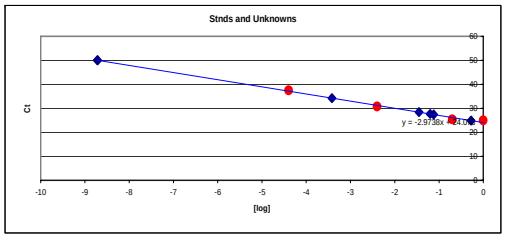
#DIV/0! Efficiency

DATA INTERFACE AND INITIAL PRE-ANALYSIS REGION …

TEST PLATE ANALYSIS FILE CONTINUED …

Log of Qty	Ct	
0	24.94	200
-0.69897	25.35	1000
-2.39794	30.78	50000
-4.39794	37.51	5000000
-8.718572	50	38.46154
-8.718572	50	50
-1.202846	27.65	100
-0.274729	24.89	500
-1.118777	27.4	5000
-1.451689	28.39	10000
-3.418892	34.24	500000

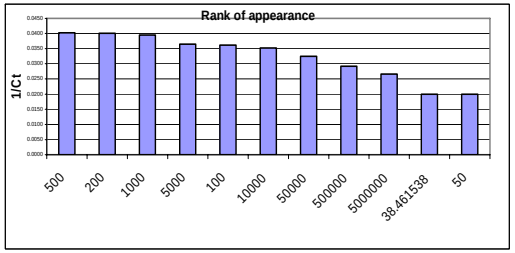
G1 Graph of Unknowns on-top of Stnd Curve:



◆ = stnd points
◆ = unknown points

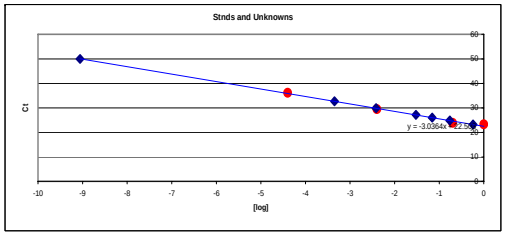
Macro: Ctrl s for sort

38.46154	50	500	24.89	0.0402
50	50	200	24.94	0.0401
100	27.65	1000	25.35	0.0394
200	24.94	5000	27.4	0.0365
500	24.89	100	27.65	0.0362
1000	25.35	10000	28.39	0.0352
5000	27.4	50000	30.78	0.0325
10000	28.39	500000	34.24	0.0292
50000	30.78	5000000	37.51	0.0267
500000	34.24	38.46154	50	0.0200
5000000	37.51	50	50	0.0200



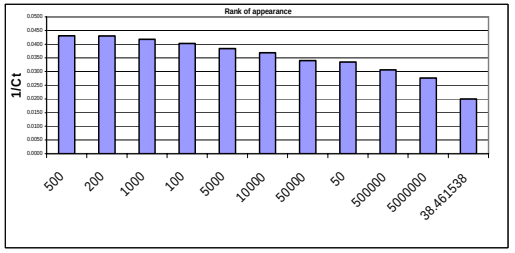
Log of Qty	Ct	
0	23.27	200
-0.69897	23.9	1000
-2.39794	29.44	50000
-4.39794	36.16	5000000
-9.055808	50	38.46154
-2.416301	29.84	50
-0.763011	24.82	100
-0.23936	23.23	500
-1.15822	26.02	5000
-1.520495	27.12	10000
-3.348335	32.67	500000

G2 Graph of Unknowns on-top of Stnd Curve:



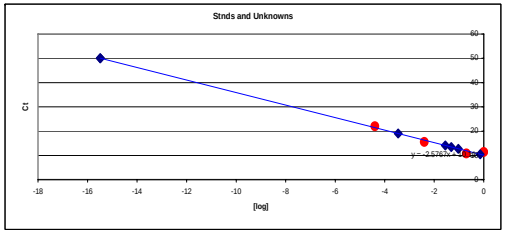
◆ = stnd points
◆ = unknown points

38.46154	50	500	23.23	0.0430
50	29.84	200	23.27	0.0430
100	24.82	1000	23.9	0.0418
200	23.27	100	24.82	0.0403
500	23.23	5000	26.02	0.0384
1000	23.9	10000	27.12	0.0369
5000	26.02	50000	29.44	0.0340
10000	27.12	50	29.84	0.0335
50000	29.44	500000	32.67	0.0306
500000	32.67	5000000	36.16	0.0277
5000000	36.16	38.46154	50	0.0200



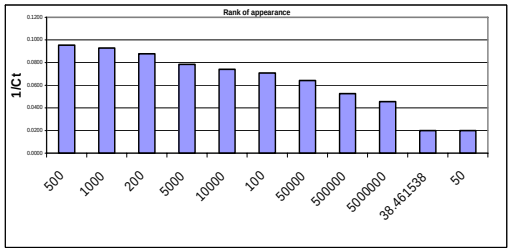
Log of Qty	Ct	
0	11.4	200
-0.69897	10.77	1000
-2.39794	15.56	50000
-4.39794	22.02	5000000
-15.4813	50	38.46154
-15.4813	50	50
-1.552564	14.11	100
-0.14766	10.49	500
-1.024754	12.75	5000
-1.315826	13.5	10000
-3.458111	19.02	500000

chR18S Graph of Unknowns on-top of Stnd Curve:



◆ = stnd points
◆ = unknown points

38.46154	50	500	10.49	0.0953
50	50	1000	10.77	0.0929
100	14.11	200	11.4	0.0877
200	11.4	5000	12.75	0.0784
500	10.49	10000	13.5	0.0741
1000	10.77	100	14.11	0.0709
5000	12.75	50000	15.56	0.0643
10000	13.5	500000	19.02	0.0526
50000	15.56	5000000	22.02	0.0454
500000	19.02	38.46154	50	0.0200
5000000	22.02	50	50	0.0200

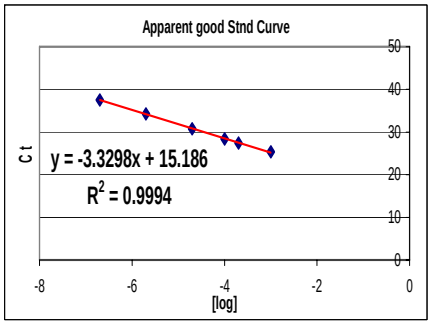


INITIAL DATA SORTING REGION
(GUIDED BY MACROS FOR QUICK NUMERIC MANIPULATIONS)

TEST PLATE ANALYSIS FILE CONTINUED …

Ctrl f (sort) — Select the points to Use here

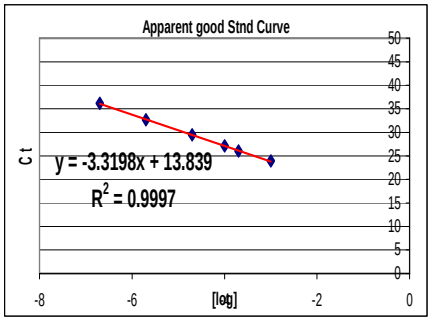
Efficiencies demonstrated	ΔCt away from ideal															
G1			50	38.461538			1000	1	0.001	-3	25.35	Efficiencies				
#DIV/0!	0.38		50	50			5000	5	0.0002	-3.69897	27.4	119.26%				
-3.05%	23.35		27.65	100			10000	10	0.0001	-4	28.39	101.41%				
-22.57%	3.71	OK	24.94	200	nope		50000	50	0.00002	-4.69897	30.78	96.09%	30.78			
-100.00%	1.37	OK	24.89	500	maybe OK?		500000	500	0.000002	-5.69897	34.24	94.54%	34.24			
351.25%	0.54	OK	25.35	1000	maybe OK?	.	1000	5000000	5000	0.0000002	-6.69897	37.51	102.21%	37.51		
119.26%	0.27	OK	27.4	5000	maybe OK?	.	5000									
101.41%	0.01	OK	28.39	10000	maybe OK?	.	10000									
96.09%	-0.07	OK	30.78	50000	maybe OK?	.	50000									
94.54%	-0.14	OK	34.24	500000	maybe OK?	.	500000									
102.21%	0.05	OK	37.51	5000000	maybe OK?	.	5000000									



G1		Stnd. Curve "in-well"
	suggested	
start useful stnd curve at 1:	1000	1000
serial 1:	10	10000
Efficiency: 99.67%	119.26% (Better E)	100000
Correlation: -1.000		1000000
b = 15.186		
m = -3.3298		

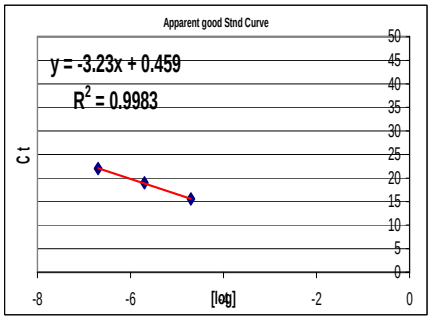
Ctrl+Shift+B to run both files

Efficiencies demonstrated	ΔCt away from ideal										log input	Ct				
G2			50	38.461538			1000	1	0.001	-3	23.9	Efficiencies				
-1.29%	20.54		29.84	50			5000	5	0.0002	-3.69897	26.02	113.65%				
-12.90%	6.02		24.82	100			10000	10	0.0001	-4	27.12	87.79%				
-36.06%	2.55	OK	23.27	200	nope		50000	50	0.00002	-4.69897	29.44	100.12%	29.44			
-100.00%	1.36	OK	23.23	500	maybe OK?		500000	500	0.000002	-5.69897	32.67	103.98%	32.67			
181.38%	0.33	OK	23.9	1000	maybe OK?	.	1000	5000000	5000	0.0000002	-6.69897	36.16	93.43%	36.16		
113.65%	0.20	OK	26.02	5000	maybe OK?	.	5000									
87.79%	-0.10	OK	27.12	10000	maybe OK?	.	10000									
100.12%	0.00	OK	29.44	50000	maybe OK?	.	50000									
103.98%	0.09	OK	32.67	500000	maybe OK?	.	500000									
93.43%	-0.17	OK	36.16	5000000	maybe OK?	.	5000000									



G2		Stnd. Curve "in-well"
	suggested	
start useful stnd curve at 1:	1000	1000
serial 1:	10	10000
Efficiency: 100.09%	113.65% (Better E)	100000
Correlation: -1.000		1000000
b = 13.839		
m = -3.3198		

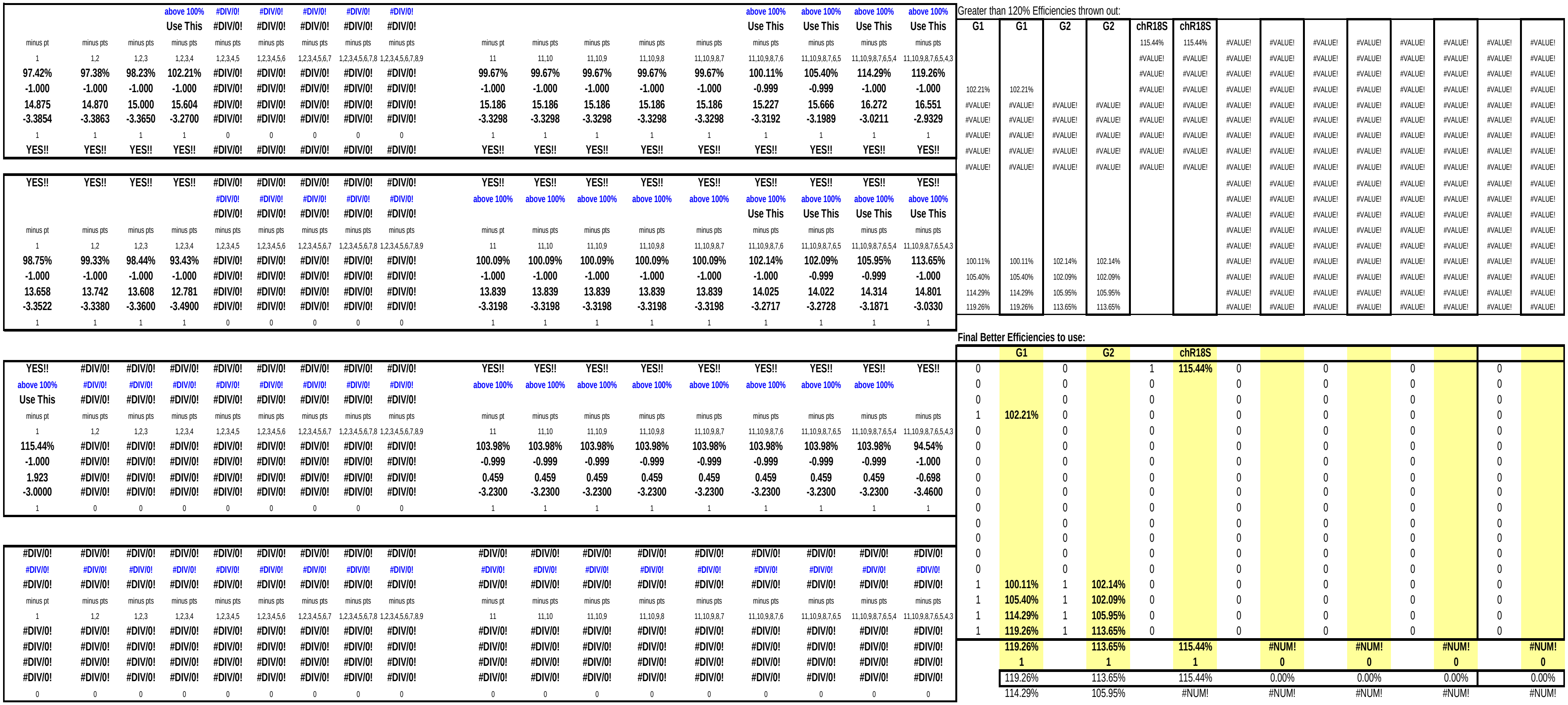
Efficiencies demonstrated	ΔCt away from ideal										log input	Ct				
chR18S			50	38.461538			50000	1	0.00002	-4.69897	15.56	Efficiencies				
#DIV/0!	0.38		50	50			500000	10	0.000002	-5.69897	19.02	94.54%				
-1.91%	36.89		14.11	100			5000000	100	0.0000002	-6.69897	22.02	115.44%				
-22.57%	3.71	OK	11.4	200	nope											
-63.47%	2.23	OK	10.49	500	maybe OK?											
1088.80%	0.72	OK	10.77	1000	maybe OK?											
125.43%	0.34	OK	12.75	5000	maybe OK?											
151.98%	0.25	OK	13.5	10000	maybe OK?											
118.43%	0.26	OK	15.56	50000	maybe OK?	.	50000									
94.54%	-0.14	OK	19.02	500000	maybe OK?	.	500000									
115.44%	0.32	OK	22.02	5000000	maybe OK?	.	5000000									



chR18S		Stnd. Curve "in-well"
	suggested	
start useful stnd curve at 1:	50000	50000
serial 1:	5	250000
Efficiency: 103.98%	115.44% (Better E)	1250000
Correlation: -0.999		6250000
b = 0.459		
m = -3.2300		

FINAL DATA SORTING REGIONS
(GUIDED BY MACROS FOR QUICK NUMERIC MANIPULATIONS)

FINAL DATA FIELD PORTION OF THE FILE FOR IDENTIFYING ANY OTHER COMBINATIONS OF POINTS WHICH WOULD YIELD BETTER EFFICIENCIES FOR EACH TARGET …



			above 100%	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!						above 100%	above 100%	above 100%	above 100%
			Use This	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!						Use This	Use This	Use This	Use This
minus pt	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pt	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts
1	1,2	1,2,3	1,2,3,4	1,2,3,4,5	1,2,3,4,5,6	1,2,3,4,5,6,7	1,2,3,4,5,6,7,8	1,2,3,4,5,6,7,8,9	11	11,10	11,10,9	11,10,9,8	11,10,9,8,7	11,10,9,8,7,6	11,10,9,8,7,6,5	11,10,9,8,7,6,5,4	11,10,9,8,7,6,5,4,3
97.42%	97.38%	98.23%	102.21%	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	99.67%	99.67%	99.67%	99.67%	99.67%	100.11%	105.40%	114.29%	119.26%
-1.000	-1.000	-1.000	-1.000	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	-1.000	-1.000	-1.000	-1.000	-1.000	-0.999	-0.999	-1.000	-1.000
14.875	14.870	15.000	15.604	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	15.186	15.186	15.186	15.186	15.186	15.227	15.666	16.272	16.551
-3.3854	-3.3863	-3.3650	-3.2700	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	-3.3298	-3.3298	-3.3298	-3.3298	-3.3298	-3.3192	-3.1989	-3.0211	-2.9329
1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1
YES!!	YES!!	YES!!	YES!!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!

YES!!	YES!!	YES!!	YES!!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!
				#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	above 100%	above 100%	above 100%	above 100%	above 100%	above 100%	above 100%	above 100%	above 100%
				#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!						Use This	Use This	Use This	Use This
minus pt	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pt	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts
1	1,2	1,2,3	1,2,3,4	1,2,3,4,5	1,2,3,4,5,6	1,2,3,4,5,6,7	1,2,3,4,5,6,7,8	1,2,3,4,5,6,7,8,9	11	11,10	11,10,9	11,10,9,8	11,10,9,8,7	11,10,9,8,7,6	11,10,9,8,7,6,5	11,10,9,8,7,6,5,4	11,10,9,8,7,6,5,4,3
98.75%	99.33%	98.44%	93.43%	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	100.09%	100.09%	100.09%	100.09%	100.09%	102.14%	102.09%	105.95%	113.65%
-1.000	-1.000	-1.000	-1.000	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	-1.000	-1.000	-1.000	-1.000	-1.000	-1.000	-0.999	-0.999	-1.000
13.658	13.742	13.608	12.781	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	13.839	13.839	13.839	13.839	13.839	14.025	14.022	14.314	14.801
-3.3522	-3.3380	-3.3600	-3.4900	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	-3.3198	-3.3198	-3.3198	-3.3198	-3.3198	-3.2717	-3.2728	-3.1871	-3.0330
1	1	1	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1

YES!!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!	YES!!
above 100%	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	above 100%	above 100%	above 100%	above 100%	above 100%	above 100%	above 100%	above 100%	
Use This	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!									
minus pt	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pt	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts
1	1,2	1,2,3	1,2,3,4	1,2,3,4,5	1,2,3,4,5,6	1,2,3,4,5,6,7	1,2,3,4,5,6,7,8	1,2,3,4,5,6,7,8,9	11	11,10	11,10,9	11,10,9,8	11,10,9,8,7	11,10,9,8,7,6	11,10,9,8,7,6,5	11,10,9,8,7,6,5,4	11,10,9,8,7,6,5,4,3
115.44%	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	103.98%	103.98%	103.98%	103.98%	103.98%	103.98%	103.98%	103.98%	94.54%
-1.000	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	-0.999	-0.999	-0.999	-0.999	-0.999	-0.999	-0.999	-0.999	-1.000
1.923	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	0.459	0.459	0.459	0.459	0.459	0.459	0.459	0.459	-0.698
-3.0000	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	-3.2300	-3.2300	-3.2300	-3.2300	-3.2300	-3.2300	-3.2300	-3.2300	-3.4600
1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1

#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
minus pt	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pt	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts	minus pts
1	1,2	1,2,3	1,2,3,4	1,2,3,4,5	1,2,3,4,5,6	1,2,3,4,5,6,7	1,2,3,4,5,6,7,8	1,2,3,4,5,6,7,8,9	11	11,10	11,10,9	11,10,9,8	11,10,9,8,7	11,10,9,8,7,6	11,10,9,8,7,6,5	11,10,9,8,7,6,5,4	11,10,9,8,7,6,5,4,3
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Greater than 120% Efficiencies thrown out:

G1	G1	G2	G2	chR18S	chR18S								
				115.44%	115.44%	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
				#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
				#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
102.21%	102.21%			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
						#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
						#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
						#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
						#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
						#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
100.11%	100.11%	102.14%	102.14%			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
105.40%	105.40%	102.09%	102.09%			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
114.29%	114.29%	105.95%	105.95%			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!
119.26%	119.26%	113.65%	113.65%			#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!	#VALUE!

Final Better Efficiencies to use:

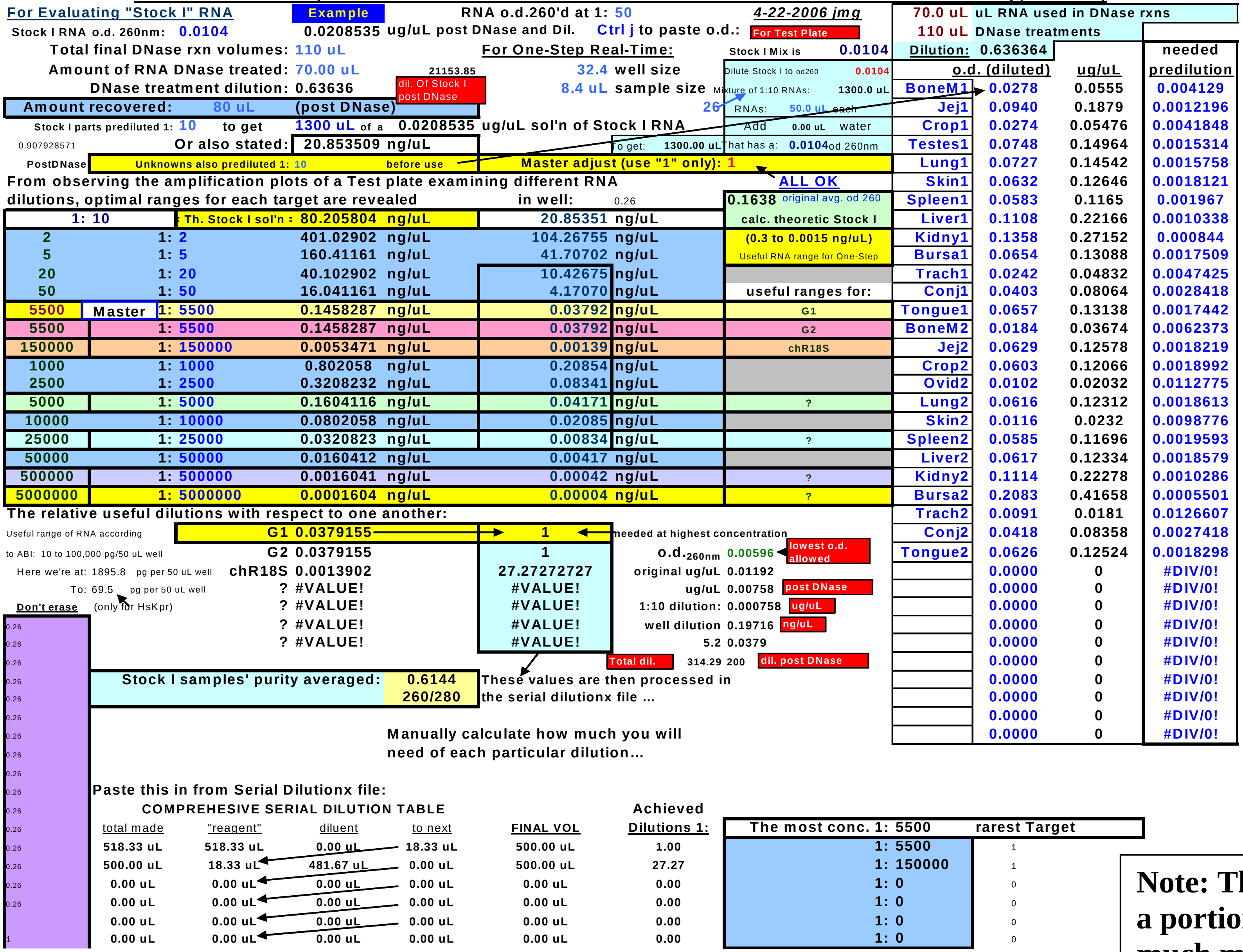
	G1		G2		chR18S								
0		0		1	115.44%	0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
1	102.21%	0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
0		0		0		0		0		0		0	
1	100.11%	1	102.14%	0		0		0		0		0	
1	105.40%	1	102.09%	0		0		0		0		0	
1	114.29%	1	105.95%	0		0		0		0		0	
1	119.26%	1	113.65%	0		0		0		0		0	
	119.26%		113.65%		115.44%		#NUM!		#NUM!		#NUM!		#NUM!
	1		1		1		0		0		0		0
	119.26%		113.65%		115.44%		0.00%		0.00%		0.00%		0.00%
	114.29%		105.95%		#NUM!		#NUM!		#NUM!		#NUM!		#NUM!

(I'VE SHOWN THIS PARTICULAR FILE IN FULL JUST TO GIVE THE READER A BETTER IDEA OF JUST HOW EXTENSIVE EACH OF THESE EXCEL FILES REALLY ARE; AND PLEASE BEAR IN MIND THAT WHAT IS STILL NOT SHOWN HERE ARE ALL THE EQUATIONS THAT HAVE BEEN ENTERED INTO EACH ACTIVE CELL SO THE FILE CAN ACTUALLY WORK AS INTENDED)

~POST TEST PLATE CALCULATIONS~

TEST PLATE RESULTS ARE THEN ENTERED INTO ANOTHER SERIES OF EXCEL FILES WHICH USE WHAT WAS LEARNED FROM THE TEST PLATE TO CALCULATE THE FINAL, OPTIMAL DILUTIONS FOR EACH RNA SAMPLE ACCORDING TO EACH TARGET INVESTIGATED. HERE, RNAs TO BE USED FOR G1 AND G2 qPCR DETERMINATIONS HAD IDENTICAL USEFUL RANGES WHILE THE RNAs TO BE USED FOR CHICKEN 18S RNA REQUIRED MUCH HIGHER DILUTIONS BEFORE USEFULNESS WAS ATTAINED …

Originally all numbers at this point were calculated an exact factor of 0.26 off – so from here on, I've re-worked the files to reflect this (i.e. 0.0104 is used instead of 0.0401 for the calculated Stock I 1:10 o.d. @ 260nm)



For Evaluating "Stock I" RNA   Example   RNA o.d.260'd at 1: 50   4-22-2006 jmg

Stock I RNA o.d. 260nm: 0.0104   0.0208535 ug/uL post DNase and Dil.   Ctrl j to paste o.d.: For Test Plate

Total final DNase rxn volumes: 110 uL

Amount of RNA DNase treated: 70.00 uL   21153.85

DNase treatment dilution: 0.63636   dil. Of Stock I post DNase

Amount recovered: 80 uL (post DNase)

Stock I parts prediluted 1: 10 to get 1300 uL of a 0.0208535 ug/uL sol'n of Stock I RNA

0.907928571   Or also stated: 20.853509 ng/uL

PostDNase   Unknowns also prediluted 1: 10 before use   Master adjust (use "1" only): 1

For One-Step Real-Time:

32.4 well size

8.4 uL sample size

26

Stock I Mix is 0.0104

Dilute Stock I to od260 0.0104

Mixture of 1:10 RNAs: 1300.0 uL

RNAs: 50.0 uL each

Add 0.00 uL water

To get: 1300.00 uL That has a: 0.0104 od 260nm

ALL OK

70.0 uL uL RNA used in DNase rxns

110 uL DNase treatments

Dilution: 0.636364

From observing the amplification plots of a Test plate examining different RNA dilutions, optimal ranges for each target are revealed

in well: 0.26

0.1638 original avg. od 260

1: 10		Th. Stock I sol'n = 80.205804 ng/uL	20.85351 ng/uL	calc. theoretic Stock I
2	1: 2	401.02902 ng/uL	104.26755 ng/uL	(0.3 to 0.0015 ng/uL)
5	1: 5	160.41161 ng/uL	41.70702 ng/uL	Useful RNA range for One-Step
20	1: 20	40.102902 ng/uL	10.42675 ng/uL	
50	1: 50	16.041161 ng/uL	4.17070 ng/uL	useful ranges for:
5500 Master	1: 5500	0.1458287 ng/uL	0.03792 ng/uL	G1
5500	1: 5500	0.1458287 ng/uL	0.03792 ng/uL	G2
150000	1: 150000	0.0053471 ng/uL	0.00139 ng/uL	chR18S
1000	1: 1000	0.802058 ng/uL	0.20854 ng/uL	
2500	1: 2500	0.3208232 ng/uL	0.08341 ng/uL	
5000	1: 5000	0.1604116 ng/uL	0.04171 ng/uL	?
10000	1: 10000	0.0802058 ng/uL	0.02085 ng/uL	
25000	1: 25000	0.0320823 ng/uL	0.00834 ng/uL	?
50000	1: 50000	0.0160412 ng/uL	0.00417 ng/uL	
500000	1: 500000	0.0016041 ng/uL	0.00042 ng/uL	?
5000000	1: 5000000	0.0001604 ng/uL	0.00004 ng/uL	?

	o.d. (diluted)	ug/uL	needed predilution
BoneM1	0.0278	0.0555	0.004129
Jej1	0.0940	0.1879	0.0012196
Crop1	0.0274	0.05476	0.0041848
Testes1	0.0748	0.14964	0.0015314
Lung1	0.0727	0.14542	0.0015758
Skin1	0.0632	0.12646	0.0018121
Spleen1	0.0583	0.1165	0.001967
Liver1	0.1108	0.22166	0.0010338
Kidny1	0.1358	0.27152	0.000844
Bursa1	0.0654	0.13088	0.0017509
Trach1	0.0242	0.04832	0.0047425
Conj1	0.0403	0.08064	0.0028418
Tongue1	0.0657	0.13138	0.0017442
BoneM2	0.0184	0.03674	0.0062373
Jej2	0.0629	0.12578	0.0018219
Crop2	0.0603	0.12066	0.0018992
Ovid2	0.0102	0.02032	0.0112775
Lung2	0.0616	0.12312	0.0018613
Skin2	0.0116	0.0232	0.0098776
Spleen2	0.0585	0.11696	0.0019593
Liver2	0.0617	0.12334	0.0018579
Kidny2	0.1114	0.22278	0.0010286
Bursa2	0.2083	0.41658	0.0005501
Trach2	0.0091	0.0181	0.0126607
Conj2	0.0418	0.08358	0.0027418
Tongue2	0.0626	0.12524	0.0018298
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!
	0.0000	0	#DIV/0!

The relative useful dilutions with respect to one another:

Useful range of RNA according to ABI: 10 to 100,000 pg/50 uL well

Here we're at: 1895.8 pg per 50 uL well

To: 69.5 pg per 50 uL well

Don't erase (only for HsKpr)

G1	0.0379155	1
G2	0.0379155	1
chR18S	0.0013902	27.27272727
?	#VALUE!	#VALUE!
?	#VALUE!	#VALUE!
?	#VALUE!	#VALUE!
?	#VALUE!	#VALUE!

needed at highest concentration

o.d.260nm 0.00596 lowest o.d. allowed

original ug/uL 0.01192

ug/uL 0.00758 post DNase

1:10 dilution: 0.000758 ug/uL

well dilution 0.19716 ng/uL

5.2 0.0379

Total dil. 314.29 200 dil. post DNase

Stock I samples' purity averaged: 0.6144 260/280

These values are then processed in the serial dilutionx file …

Manually calculate how much you will need of each particular dilution…

0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
0.26
1

Paste this in from Serial Dilutionx file:

COMPREHESIVE SERIAL DILUTION TABLE

total made	"reagent"	diluent	to next	FINAL VOL	Achieved Dilutions 1:
518.33 uL	518.33 uL	0.00 uL	18.33 uL	500.00 uL	1.00
500.00 uL	18.33 uL	481.67 uL	0.00 uL	500.00 uL	27.27
0.00 uL	0.00 uL	0.00 uL	0.00 uL	0.00 uL	0.00
0.00 uL	0.00 uL	0.00 uL	0.00 uL	0.00 uL	0.00
0.00 uL	0.00 uL	0.00 uL	0.00 uL	0.00 uL	0.00
0.00 uL	0.00 uL	0.00 uL	0.00 uL	0.00 uL	0.00

The most conc. 1: 5500	rarest Target
1: 5500	1
1: 150000	1
1: 0	0
1: 0	0
1: 0	0
1: 0	0

Note: This is only a portion of a much more extensive file

THIS FILE PROVIDES ANOTHER OVERVIEW OF WHAT WE ARE ACCOMPLISHING HERE:

Post-DNased, 1:10-diluted samples

Each 1:10 sample made: 800 uL
used for Stock I: 50 uL

"Tier 1 Dilutions"
For initial qPCR sample target dilution (e.g.):

Tier 1 concentration achieved in-well ng/uL: 0.03792

	od 260's	ng/uL post DNase predilution 0.1	ng/uL Additional predilution needed 1:	ng/uL in-well dilution incurred 0.26		post DNase 1:	Inhibition?	Use of sample:	nuc-free water	uL Left
1	0.2775	35.32	242.19	9.18	Dilution possible	9314.98	OK	4.2 uL	1014.13 uL	746 uL
2	0.9395	119.57	819.95	31.09	Dilution possible	31536.66	OK	1.2 uL	1017.09 uL	749 uL
3	0.2738	34.85	238.96	9.06	Dilution possible	9190.78	OK	4.3 uL	1014.07 uL	746 uL
4	0.7482	95.23	653.00	24.76	Dilution possible	25115.20	OK	1.6 uL	1016.77 uL	748 uL
5	0.7271	92.54	634.58	24.06	Dilution possible	24406.92	OK	1.6 uL	1016.73 uL	748 uL
6	0.6323	80.47	551.84	20.92	Dilution possible	21224.73	OK	1.8 uL	1016.49 uL	748 uL
7	0.5825	74.14	508.38	19.28	Dilution possible	19553.06	OK	2.0 uL	1016.33 uL	748 uL
8	1.1083	141.06	967.27	36.67	Dilution possible	37202.85	OK	1.1 uL	1017.28 uL	749 uL
9	1.3576	172.79	1184.85	44.92	Dilution possible	45571.23	OK	0.9 uL	1017.47 uL	749 uL
10	0.6544	83.29	571.13	21.65	Dilution possible	21966.57	OK	1.8 uL	1016.55 uL	748 uL
11	0.2416	30.75	210.86	7.99	Dilution possible	8109.91	OK	4.8 uL	1013.50 uL	745 uL
12	0.4032	51.32	351.89	13.34	Dilution possible	13534.41	OK	2.9 uL	1015.44 uL	747 uL
13	0.6569	83.61	573.31	21.74	Dilution possible	22050.49	OK	1.8 uL	1016.56 uL	748 uL
14	0.1837	23.38	160.33	6.08	Dilution possible	6166.35	OK	6.4 uL	1011.98 uL	744 uL
15	0.6289	80.04	548.88	20.81	Dilution possible	21110.60	OK	1.9 uL	1016.48 uL	748 uL
16	0.6033	76.78	526.53	19.96	Dilution possible	20251.27	OK	1.9 uL	1016.40 uL	748 uL
17	0.1016	12.93	88.67	3.36	Dilution possible	3410.46	OK	11.5 uL	1006.85 uL	739 uL
18	0.6156	78.35	537.27	20.37	Dilution possible	20664.15	OK	1.9 uL	1016.44 uL	748 uL
19	0.116	14.76	101.24	3.84	Dilution possible	3893.83	OK	10.1 uL	1008.27 uL	740 uL
20	0.5848	74.43	510.39	19.35	Dilution possible	19630.27	OK	2.0 uL	1016.34 uL	748 uL
21	0.6167	78.49	538.23	20.41	Dilution possible	20701.07	OK	1.9 uL	1016.44 uL	748 uL
22	1.1139	141.77	972.16	36.86	Dilution possible	37390.83	OK	1.0 uL	1017.29 uL	749 uL
23	2.0829	265.10	1817.86	68.93	Dilution possible	69917.73	OK	0.6 uL	1017.77 uL	749 uL
24	0.0905	11.52	78.98	2.99	Dilution possible	3037.86	OK	12.9 uL	1005.44 uL	737 uL
25	0.4179	53.19	364.72	13.83	Dilution possible	14027.85	OK	2.8 uL	1015.54 uL	747 uL
26	0.6262	79.70	546.52	20.72	Dilution possible	21019.96	OK	1.9 uL	1016.47 uL	748 uL

AFTER THE TEST PLATE, THE FIRST THING TO DO IS ESTABLISH THE "FIRST TIER" DILUTIONS (THOSE DILUTIONS OF RNA WHICH ARE USEFUL FOR THE TARGET OR TARGETS WHICH CAN UTILIZE THE MOST CONCENTRATED OF THE RNA DILUTIONS AS DETERMINED BY THE TEST PLATE). THIS FILE DEMONSTRATAES THE OFTEN MINISCULE AMOUNTS OF POST-DNASED, 1:10-DILUTED RNAS THAT ARE THEN FURTHER DILUTED IN ORDER TO ATTAIN THE FIRST TIER DILUTION RANGE(!) …

Using a standard RNA (containing all your targets of interest), run a test plate, testing various dilutions of the RNA until it no longer exhibits template or chemical inhibition of each qPCR reaction … Decide where your standard curves should start for each target (e.g. always after the point of template or chemical inhibition for each target) …

FROM YOUR OBSERVATIONS OF THE TEST PLATE RESULTS, DECIDE THE FOLLOWING:

(Decide what target needs the most concentrated RNA in order to be found adequately by qPCR. Enter that target and its apparent 1st useful dilution in the yellow area below):

List in order of abundance from weakest to strongest (as observed from Cts on your Test Plate)

TEST PLATE OBSERVATIONS

(least abundant target: "limiting" factor)

Have to have at least 3 samples here, and for any samples that share identical dilutions, be sure to alter 1 of them slightly -- i.e. 1:300 and 1:300.000001 etc. -- or else the file will not work

		Enter values of apparent 1st useful dilution 1:	This serves as the 1st point (dilution) in the standard curve for this target ng/uL (in-well) that this dilution actually corresponds to (info from Sheet 3 used)	Apparent useful serial dilution factor for each Stnd Crve 1:
Target 1	G1	1000	0.2085 ng/uL in-well	10
Target 2	G2	1000	0.2085 ng/uL in-well	10
Target 3	chR18S	50000	0.0042 ng/uL in-well	5
Target 4	?	?		?
Target 5	?	?		?
Target 6	?	?		?
Target 7	?	?		?

4 points

G1	G2	chR18S	?	?	?	?
1000	1000	50000	?	?	?	?
10000	10000	250000	?	?	?	?
100000	100000	1250000	?	?	?	?
1000000	1000000	6250000	?	?	?	?

Or, in final ng/uL (in well values):

G1	G2	chR18S	?	?	?	?
0.2085	0.2085	0.0042	0.0000	0.0000	0.0000	0.0000
0.0209	0.0209	0.0008	0.0000	0.0000	0.0000	0.0000
0.0021	0.0021	0.0002	0.0000	0.0000	0.0000	0.0000
0.000209	0.000209	0.000033	0.000000	0.000000	0.000000	0.000000

chR18S	To fit within stnd curve:	Name	Apparent useful Unknown dilution 1:	(in-well)			
	Target 1	G1	5500	0.03792 ng/uL in-well			
	Target 2	G2	5500	0.03792 ng/uL in-well	1	1	
x	Target 3	chR18S	150000	0.00139 ng/uL in-well	27.27272727	27.27272727	27

Type into 5700 as factors for Stnd curve relative dilutions:

G-1	G-2	ch18S
1	1	1
0.1	0.1	0.2
0.01	0.01	0.04
0.001	0.001	0.008

*i.e. sample unknowns are still "unknowns" – and they will be what they will be; but, since the Stock I mixture (to which we have calibrated this entire study; for each target) was a mixture of all unknowns, we have exponentially increased the probability that most of our unknowns will fall within the accurate portion of each target's useful standard curve. Take some time to sit back and think about this… it is imperative that the researcher understand this intimately.

EXTREMELY IMPORTANT TO READ!

This file ensures that all experimental samples are diluted to generate qPCR signals which will *theoretically appear between the first two points of each target's respective standard curve (*regardless of whether they will or not – however, this consideration is learned from the Test Plate and is applied responsibly here). The guiding parameter gleaned from the Test Plate (and used here) is how many ng of RNA must be used per uL in the final qPCR reactions per target investigated (since this feature is differentially optimal from target to target – as the Test Plate has also shown), e.g. here, for G1 and G2, 0.03792 ng total RNA/uL of reaction mixture should put all samples within the range where they can be expected to amplify without inhibition for G1 and G2 while also having the best chance of amplifying within the early portion of their respective standard curves. Sample RNAs interrogated for the presence of Chicken 18S rRNA however, must be used at 0.00139 ng total RNA/uL of reaction mixture to attain the same end in this regard. This once again illustrates the importance of running a Test Plate as described in this study; it reveals all the important parameters one will use throughout the remainder of the qPCR experiment. And, better yet, the RNA sample which is examined by the Test Plate is made up of all the experimental samples themselves – and this controls for any and all perturbatory artifacts which may have resulted from the RNA isolations themselves. In other words, we have successfully formed a "closed system" by using the experimental samples themselves as the measure against which all final qPCR signals are weighed; no exogenous elements are introduced by using a foreign source of RNA from which to prepare standards and calibrators (like others do; i.e. plasmids etc.): THIS IS SO IMPORTANT!

KEEPING IN MIND HOW MUCH OF EACH DILUTED RNA WILL BE REQUIRED TO SET UP ALL PLATES, THIS PORTION OF THE ABOVE FILE NETWORK CALCULATES THE **VOLUME** REQUIREMENTS NECESSARY TO OBTAIN ALL FINAL SAMPLE RNA DILUTIONS:

PROGRESSIVE SERIAL DILUTION WORKSHEET

jmg/6-9-2003

This program allows one to make up to 20 serial dilutions using the smallest possible amount of starting reagent

+d after adjusted

(Print out p. 1 or pp. 2, 3 and 4 when finished)

ume Adjust 1X

#DIV/0! **mL** (use this value for volume -- to use at least 1 uL of starting reagent)

DONE!

Use
: Type in
dilutions
as the
ated
mber "1"
cells
t using
culations
: Type
nal
ch res-
olutions
used cells
lculation:
trl+d
and use
, or
2, 3 & 4,
ages you
lab ...

	Adjust Column 1 values to desired dilutions (type in "1" otherwise)	Column 1: Desired final ending dilution values	Column 2: Desired Final Vol.s (mL)		Adjustable Total Starting Volumes	Original ng/mL 5400
A	1:	1.000 (More dilute)	0.50	518.3 uL copy	518.3 uL	0.00
B	1:	27.273	0.50	500.0 uL copy	500.0 uL	198.00
C	1:	1.000	0.00	0.0 uL copy	0.0 uL	0.00
D	1:	1.000	0.00	0.0 uL copy	0.0 uL	0.00
E	1:	1.000	0.00	0.0 uL copy	0.0 uL	0.00
F	1:	1.000	0.00	0.0 uL copy	0.0 uL	0.00
G	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
H	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
I	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
J	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
K	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
L	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
M	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
N	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
O	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
P	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
Q	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
R	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
S	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
T	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
U	1:	1	0.00	0.0 uL copy	0.0 uL	0.00
			Total starting reagent stock needed:		0.000 uL	achieved ng/mL
			Total diluent needed for this series of dilutions:		0.482 mL	

ion dilution scenarios)

COMPREHESIVE SERIAL DILUTION TABLE

	total made	"reagent"	diluent	to next	(FINAL VOL.)	Achieved Dilutions 1:
A	518.3 uL	518.3 uL	0.0 uL	18.3 uL	500.0 uL	1.00
B	500.0 uL	18.3 uL	481.7 uL	0.0 uL	500.0 uL	27.27

THIS PORTION OF THE FILE NETWORK ALLOWS ONE TO GET ALL FINAL VOLUMES WITHIN THE RANGE WHERE THE EPPENDORF epMOTION 5070 LIQUID-HANDLING ROBOT CAN EASILY HANDLE ALL SAMPLE SERIAL DILUTIONS:

(notice that G1 and G2 use the same RNA dilution here)

(in-well) 1st sample dilutions incurred | **1st sample adjust** The Final print-out for final sample serial dilutions to get them into the appropriate useful One-Step real-time qPCR ranges without inhibiti

Status	Post DNase 1:	Since isolation 1:	1st-tier ng/uL check		0.005606263	Sample	1:10 RNA	Water	1st total	from previous	Water	from previous	Water
OK	9314.98	14637.82		1	0.0379155	BoneM1	4.2 uL	1014.13 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	31536.66	49557.61		1	0.0379155	Jej1	1.2 uL	1017.09 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	9190.78	14442.65		1	0.0379155	Crop1	4.3 uL	1014.07 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	25115.20	39466.74	1		0.0379155	Testes1	1.6 uL	1016.77 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	24406.92	38353.74	1		0.0379155	Lung1	1.6 uL	1016.73 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	21224.73	33353.14	1		0.0379155	Skin1	1.8 uL	1016.49 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	19553.06	30726.24	1		0.0379155	Spleen1	2.0 uL	1016.33 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	37202.85	58461.62	1		0.0379155	Liver1	1.1 uL	1017.28 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	45571.23	71611.93	1		0.0379155	Kidny1	0.9 uL	1017.47 uL	1018 uL	518.33 uL	0.00		481.67 uL
OK	21966.57	34518.89	1		0.0379155	Bursa1	1.8 uL	1016.55 uL	1018 uL	518.33 uL	0.00		481.67 uL
OK	8109.91	12744.14	1		0.0379155	Trach1	4.8 uL	1013.50 uL	1018 uL	518.33 uL	0.00		481.67 uL
OK	13534.41	21268.36	1		0.0379155	Conj1	2.9 uL	1015.44 uL	1018 uL	518.33 uL	0.00		481.67 uL
OK	22050.49	34650.76	1		0.0379155	Tongue1	1.8 uL	1016.56 uL	1018 uL	518.33 uL	0.00		481.67 uL
OK	6166.35	9689.98	1		0.0379155	BoneM2	6.4 uL	1011.98 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	21110.60	33173.79	1		0.0379155	Jej2	1.9 uL	1016.48 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	20251.27	31823.42	1		0.0379155	Crop2	1.9 uL	1016.40 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	3410.46	5359.29	1		0.0379155	Ovid2	11.5 uL	1006.85 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	20664.15	32472.23	1		0.0379155	Lung2	1.9 uL	1016.44 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	3893.83	6118.87	1		0.0379155	Skin2	10.1 uL	1008.27 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	19630.27	30847.57	1		0.0379155	Spleen2	2.0 uL	1016.34 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	20701.07	32530.26	1		0.0379155	Liver2	1.9 uL	1016.44 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	37390.83	58757.02	1		0.0379155	Kidny2	1.0 uL	1017.29 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	69917.73	109870.72	1		0.0379155	Bursa2	0.6 uL	1017.77 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	3037.86	4773.78	1		0.0379155	Trach2	12.9 uL	1005.44 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	14027.85	22043.77	1		0.0379155	Conj2	2.8 uL	1015.54 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL
OK	21019.96	33031.37	1		0.0379155	Tongue2	1.9 uL	1016.47 uL	1018 uL	518.33 uL	0.00 uL	18.33 uL	481.67 uL

These values are rounded to the nearest uL to accommodate the 5070 robot (i.e. 18 uL and 480 uL here)

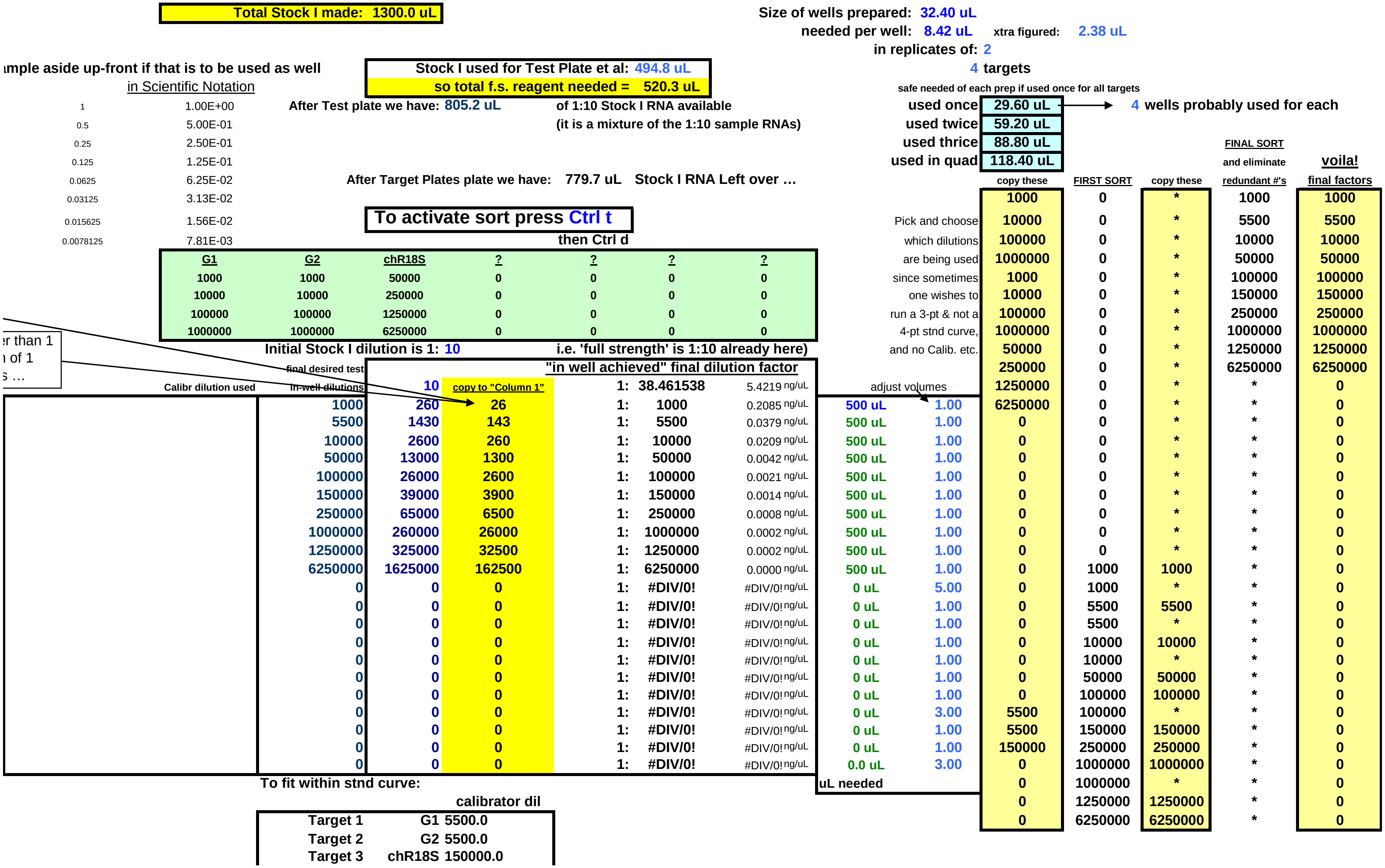
(PORTIONS OF THESE NUMBERS HAVE ALREADY BEEN SHOWN IN A FILE ABOVE)

THIS FILE ALSO DESCRIBES WHAT FINAL OVER-ALL DILUTIONS HAVE BEEN INCURRED BY EACH SAMPLE SINCE THEIR ISOLATION, AND SINCE THEIR DNAse TREATMENTS … (IT IS IMPORTANT TO KNOW THAT POST-DNase TREATMENT DILUTIONS ALL EXCEED 1:200 MINIMUM)

THIS PORTION OF THE FILE NETWORK SHOWS THE FINAL VOLUMES ATTAINED OF EACH RNA SAMPLE DILUTION WHICH ARE AVAILABLE AS THE SAMPLES USED ON THE FINAL PLATES:

final volumes Sample	optimal for Target 1 G1	optimal for Target 2 G2	optimal for Target 3 chR18S
BoneM1	500.00 uL	500.00 uL	500.00 uL
Jej1	500.00 uL	500.00 uL	500.00 uL
Crop1	500.00 uL	500.00 uL	500.00 uL
Testes1	500.00 uL	500.00 uL	500.00 uL
Lung1	500.00 uL	500.00 uL	500.00 uL
Skin1	500.00 uL	500.00 uL	500.00 uL
Spleen1	500.00 uL	500.00 uL	500.00 uL
Liver1	500.00 uL	500.00 uL	500.00 uL
Kidny1	500.00 uL	500.00 uL	500.00 uL
Bursa1	500.00 uL	500.00 uL	500.00 uL
Trach1	500.00 uL	500.00 uL	500.00 uL
Conj1	500.00 uL	500.00 uL	500.00 uL
Tongue1	500.00 uL	500.00 uL	500.00 uL
BoneM2	500.00 uL	500.00 uL	500.00 uL
Jej2	500.00 uL	500.00 uL	500.00 uL
Crop2	500.00 uL	500.00 uL	500.00 uL
Ovid2	500.00 uL	500.00 uL	500.00 uL
Lung2	500.00 uL	500.00 uL	500.00 uL
Skin2	500.00 uL	500.00 uL	500.00 uL
Spleen2	500.00 uL	500.00 uL	500.00 uL
Liver2	500.00 uL	500.00 uL	500.00 uL
Kidny2	500.00 uL	500.00 uL	500.00 uL
Bursa2	500.00 uL	500.00 uL	500.00 uL
Trach2	500.00 uL	500.00 uL	500.00 uL
Conj2	500.00 uL	500.00 uL	500.00 uL
Tongue2	500.00 uL	500.00 uL	500.00 uL

THIS SERIAL DILUTION FILE INSTRUCTS US HOW TO DILUTE OUR STOCK I SAMPLE INTO THE REGION WHICH EXACTLY INCLUDES THE ENTIRE DILUTION RANGE WITHIN WHICH THE OPTIMAL RANGES FOR ALL TARGETS' STANDARD CURVES AND THEIR RESPECTIVE INTER-PLATE CALIBRATOR SAMPLES EXIST – AS INDICATED TO US BY THE TEST PLATE'S RESULTS:



Total Stock I made: 1300.0 uL

Size of wells prepared: 32.40 uL
needed per well: 8.42 uL xtra figured: 2.38 uL
in replicates of: 2
4 targets

mple aside up-front if that is to be used as well

Stock I used for Test Plate et al: 494.8 uL
so total f.s. reagent needed = 520.3 uL

	in Scientific Notation
1	1.00E+00
0.5	5.00E-01
0.25	2.50E-01
0.125	1.25E-01
0.0625	6.25E-02
0.03125	3.13E-02
0.015625	1.56E-02
0.0078125	7.81E-03

After Test plate we have: 805.2 uL of 1:10 Stock I RNA available
(it is a mixture of the 1:10 sample RNAs)

safe needed of each prep if used once for all targets

used once	29.60 uL
used twice	59.20 uL
used thrice	88.80 uL
used in quad	118.40 uL

→ 4 wells probably used for each

After Target Plates plate we have: 779.7 uL Stock I RNA Left over …

To activate sort press Ctrl t
then Ctrl d

G1	G2	chR18S	?	?	?	?
1000	1000	50000	0	0	0	0
10000	10000	250000	0	0	0	0
100000	100000	1250000	0	0	0	0
1000000	1000000	6250000	0	0	0	0

r than 1
of 1
s …

Initial Stock I dilution is 1: 10 i.e. 'full strength' is 1:10 already here)

Calibr dilution used	final desired test ~~in-well dilutions~~	10	copy to "Column 1"	"in well achieved" final dilution factor		adjust volumes	
				1: 38.461538	5.4219 ng/uL		
	1000	260	26	1: 1000	0.2085 ng/uL	500 uL	1.00
	5500	1430	143	1: 5500	0.0379 ng/uL	500 uL	1.00
	10000	2600	260	1: 10000	0.0209 ng/uL	500 uL	1.00
	50000	13000	1300	1: 50000	0.0042 ng/uL	500 uL	1.00
	100000	26000	2600	1: 100000	0.0021 ng/uL	500 uL	1.00
	150000	39000	3900	1: 150000	0.0014 ng/uL	500 uL	1.00
	250000	65000	6500	1: 250000	0.0008 ng/uL	500 uL	1.00
	1000000	260000	26000	1: 1000000	0.0002 ng/uL	500 uL	1.00
	1250000	325000	32500	1: 1250000	0.0002 ng/uL	500 uL	1.00
	6250000	1625000	162500	1: 6250000	0.0000 ng/uL	500 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	5.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	3.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0 uL	1.00
	0	0	0	1: #DIV/0!	#DIV/0! ng/uL	0.0 uL	3.00
						uL needed	

To fit within stnd curve:

		calibrator dil
Target 1	G1	5500.0
Target 2	G2	5500.0
Target 3	chR18S	150000.0

	copy these	FIRST SORT	copy these	FINAL SORT and eliminate redundant #'s	voila! final factors
	1000	0	*	1000	1000
Pick and choose	10000	0	*	5500	5500
which dilutions	100000	0	*	10000	10000
are being used	1000000	0	*	50000	50000
since sometimes	1000	0	*	100000	100000
one wishes to	10000	0	*	150000	150000
run a 3-pt & not a	100000	0	*	250000	250000
4-pt stnd curve,	1000000	0	*	1000000	1000000
and no Calib. etc.	50000	0	*	1250000	1250000
	250000	0	*	6250000	6250000
	1250000	0	*	*	0
	6250000	0	*	*	0
	0	0	*	*	0
	0	0	*	*	0
	0	0	*	*	0
	0	0	*	*	0
	0	0	*	*	0
	0	0	*	*	0
	0	0	*	*	0
	0	0	*	*	0
	0	1000	1000	*	0
	0	1000	*	*	0
	0	5500	5500	*	0
	0	5500	*	*	0
	0	10000	10000	*	0
	0	10000	*	*	0
	0	50000	50000	*	0
	0	100000	100000	*	0
	5500	100000	*	*	0
	5500	150000	150000	*	0
	150000	250000	250000	*	0
	0	1000000	1000000	*	0
	0	1000000	*	*	0
	0	1250000	1250000	*	0
	0	6250000	6250000	*	0

THIS IS ANOTHER PORTION OF THE FILE ON THE PREVIOUS PAGE:

PROGRESSIVE SERIAL DILUTION WORKSHEET

jmg/6-24-2005/8-22-05

This program allows one to make up to 20 serial dilutions using the smallest possible amount of starting reagent

Enter: Cntrl+d after adjusted

Master Volume Adjust	1X

(Print out p. 1 or pp. 2, 3 and 4 when finished)

. mL (use this value for volume -- to use at least 1 uL of starting reagent)

DONE!

Test area to show values for cor
progressive serial dilutions at a
1:
factors to enter are

How To Use

1.) In Column 1: Type in in desired final dilutions starting with A as the most concentrated

2.) Type the number "1" in all Column 1 cells that you are not using for dilution calculations

3.) In Column 2: Type in all desired final volumes for each respective serial solutions Type "0" in unused cells

4.) Activate calculation:

Enter: Cntrl+d

5.) Print page 1 and use table at bottom, or print out pages 2, 3 & 4, or just those pages you will need in the lab ...

Also:

	Adjust Column 1 values to desired dilutions (type in "1" otherwise)	Column 1: Desired final ending dilution values	Column 2: Desired Final Vol.s (mL)			Adjustable Total Starting Volumes	Original ng/mL 10000
A	1:	26.00 (More dilute)	0.500	662.2 uL	copy	662.2 uL	384.62
B	1:	143.00	0.500	892.2 uL	copy	892.2 uL	69.93
C	1:	260.00	0.500	713.1 uL	copy	713.1 uL	38.46
D	1:	1300.00	0.500	1065.7 uL	copy	1065.7 uL	7.69
E	1:	2600.00	0.500	1131.3 uL	copy	1131.3 uL	3.85
F	1:	3900.00	0.500	947.0 uL	copy	947.0 uL	2.56
G	1:	6500.00	0.500	745.0 uL	copy	745.0 uL	1.54
H	1:	26000.00	0.500	980.0 uL	copy	980.0 uL	0.38
I	1:	32500.00	0.500	600.0 uL	copy	600.0 uL	0.31
J	1:	162500.00	0.500	500.0 uL	copy	500.0 uL	0.06
K	1:	1.00	0.000	0.0 uL	copy	0.0 uL	0.00
L	1:	1.00	0.000	0.0 uL	copy	0.0 uL	0.00
M	1:	1.00	0.000	0.0 uL	copy	0.0 uL	0.00
N	1:	1.00	0.000	0.0 uL	copy	0.0 uL	0.00
O	1:	1.0	0.000	0.0 uL	copy	0.0 uL	0.00
P	1:	1.0	0.000	0.0 uL	copy	0.0 uL	0.00
Q	1:	1.0	0.000	0.0 uL	copy	0.0 uL	0.00
R	1:	1.0	0.000	0.0 uL	copy	0.0 uL	0.00
S	1:	1.0	0.000	0.0 uL	copy	0.0 uL	0.00
T	1:	1.0	0.000	0.0 uL	copy	0.0 uL	0.00
U	1:	1.0	0.000	0.0 uL	copy	0.0 uL	0.00
			Total starting reagent stock needed:			25.470 uL	achieved ng/mL
			Total diluent needed for this series of dilutions:			4.975 mL	

A	1
B	2
C	4
D	8
E	16
F	32
G	128

	Desired Fin
A	0.15
B	0.15
C	0.15
D	0.15
E	0.15
F	0.15
G	0.15

PREX

(Some common dilution scenarios)

To activate press Ctrl d

Stock I

COMPREHENSIVE SERIAL DILUTION TABLE

	total made	"reagent"	diluent	to next	(FINAL VOL.)	Achieved Dilutions 1:	Actual Final Dilutions Achieved for Final Plates after used in-well:		4-pt Standard Curves
A	662.2 uL	25.5 uL	636.8 uL	162.2 uL	500.0 uL	26.0	1: 1000	A	
B	892.2 uL	162.2 uL	730.0 uL	392.2 uL	500.0 uL	143.0	1: 5500	B	Calibrator G1 & G2
C	713.1 uL	392.2 uL	320.9 uL	213.1 uL	500.0 uL	260.0	1: 10000	C	
D	1065.7 uL	213.1 uL	852.5 uL	565.7 uL	500.0 uL	1300.0	1: 50000	D	
E	1131.3 uL	565.7 uL	565.7 uL	631.3 uL	500.0 uL	2600.0	1: 100000	E	
F	947.0 uL	631.3 uL	315.7 uL	447.0 uL	500.0 uL	3900.0	1: 150000	F	Calibrator chRIBO18S
G	745.0 uL	447.0 uL	298.0 uL	245.0 uL	500.0 uL	6500.0	1: 250000	G	
H	980.0 uL	245.0 uL	735.0 uL	480.0 uL	500.0 uL	26000.0	1: 1000000	H	
I	600.0 uL	480.0 uL	120.0 uL	100.0 uL	500.0 uL	32500.0	1: 1250000	I	
J	500.0 uL	100.0 uL	400.0 uL	0.0 uL	500.0 uL	162500.0	1: 6250000	J	

~THE PLATES~

ALL PLATES DRAWN OUT:

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	B	B	A	A	C	C	E	E	H	H
B	1	1	2	2	3	3	4	4	5	5	6	6
C	7	7	8	8	9	9	10	10	11	11	12	12
D	13	13										
E	NTC	NTC	F	F	D	D	G	G	I	I	J	J
F	1	1	2	2	3	3	4	4	5	5	6	6
G	7	7	8	8	9	9	10	10	11	11	12	12
H	13	13										

G-1 Plate 1
dilution sA
1-13

Ch18S
dilution sB
1-13

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	B	B	A	A	C	C	E	E	H	H
B	14	14	15	15	16	16	17	17	18	18	19	19
C	20	20	21	21	22	22	23	23	24	24	25	25
D	26	26										
E	NTC	NTC	F	F	D	D	G	G	I	I	J	J
F	14	14	15	15	16	16	17	17	18	18	19	19
G	20	20	21	21	22	22	23	23	24	24	25	25
H	26	26										

G-1 Plate 2
dilution sA
14-26

Ch18S
dilution sB
14-26

C

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	B	B	A	A	C	C	E	E	H	H
B	1	1	2	2	3	3	4	4	5	5	6	6
C	7	7	8	8	9	9	10	10	11	11	12	12
D	13	13										
E	NTC	NTC	F	F	D	D	G	G	I	I	J	J
F	1	1	2	2	3	3	4	4	5	5	6	6
G	7	7	8	8	9	9	10	10	11	11	12	12
H	13	13										

G-2 Plate 1
dilution sA
1-13

Ch18S
dilution sB
1-13

D

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	B	B	A	A	C	C	E	E	H	H
B	14	14	15	15	16	16	17	17	18	18	19	19
C	20	20	21	21	22	22	23	23	24	24	25	25
D	26	26										
E	NTC	NTC	F	F	D	D	G	G	I	I	J	J
F	14	14	15	15	16	16	17	17	18	18	19	19
G	20	20	21	21	22	22	23	23	24	24	25	25
H	26	26										

G-2 Plate 2
dilution sA
14-26

Ch18S
dilution sB
14-26

E

	1	2	3	4	5	6	7	8	9	10	11	12
A	F	D	G	I	J	1	2	3	4	5	6	7
B	8	9	10	11	12	13	14	15	16	17	18	19
C	20	21	22	23	24	25	26					
D												
E												
F												
G												
H												

NRC Plate 1 (18S)
dilution sB

All samples

in singlet

MANUAL TALLY OF TOTAL WELL USAGE:

Wells per target:

G-1	76
G-2	76
Ch18S	152

For NRC plates

Ch18S	31

KEY DESCRIBING WHICH TISSUE RNAs WERE ANALYZED PER WELL:

A

A	NTC	NTC	B	B	A	A	C	C	E	E	H	H
B	BneM1	BneM1	Jejun1	Jejun1	Crop1	Crop1	Testes1	Testes1	Lung1	Lung1	Skin1	Skin1
C	Spleen1	Spleen1	Liver1	Liver1	Kidny1	Kidny1	Bursa1	Bursa1	Trach1	Trach1	Conj1	Conj1
D	Tngue1	Tngue1										
E	NTC	NTC	F	F	D	D	G	G	I	I	J	J
F	BneM1	BneM1	Jejun1	Jejun1	Crop1	Crop1	Testes1	Testes1	Lung1	Lung1	Skin1	Skin1
G	Spleen1	Spleen1	Liver1	Liver1	Kidny1	Kidny1	Bursa1	Bursa1	Trach1	Trach1	Conj1	Conj1
H	Tngue1	Tngue1										

Plate for G1 and 18S for chicken 1 (male)

B

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	B	B	A	A	C	C	E	E	H	H
B	BneM2	BneM2	Jejun2	Jejun2	Crop2	Crop2	Ovid2	Ovid2	Lung2	Lung2	Skin2	Skin2
C	Spleen2	Spleen2	Liver2	Liver2	Kidny2	Kidny2	Bursa2	Bursa2	Trach2	Trach2	Conj2	Conj2
D	Tngue2	Tngue2										
E	NTC	NTC	F	F	D	D	G	G	I	I	J	J
F	BneM2	BneM2	Jejun2	Jejun2	Crop2	Crop2	Ovid2	Ovid2	Lung2	Lung2	Skin2	Skin2
G	Spleen2	Spleen2	Liver2	Liver2	Kidny2	Kidny2	Bursa2	Bursa2	Trach2	Trach2	Conj2	Conj2
H	Tngue2	Tngue2										

Plate for G1 and 18S for chicken 2 (female)

C

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	B	B	A	A	C	C	E	E	H	H
B	BneM1	BneM1	Jejun1	Jejun1	Crop1	Crop1	Testes1	Testes1	Lung1	Lung1	Skin1	Skin1
C	Spleen1	Spleen1	Liver1	Liver1	Kidny1	Kidny1	Bursa1	Bursa1	Trach1	Trach1	Conj1	Conj1
D	Tngue1	Tngue1										
E	NTC	NTC	F	F	D	D	G	G	I	I	J	J
F	BneM1	BneM1	Jejun1	Jejun1	Crop1	Crop1	Testes1	Testes1	Lung1	Lung1	Skin1	Skin1
G	Spleen1	Spleen1	Liver1	Liver1	Kidny1	Kidny1	Bursa1	Bursa1	Trach1	Trach1	Conj1	Conj1
H	Tngue1	Tngue1										

Plate for G2 and 18S for chicken 1 (male)

D

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	NTC	B	B	A	A	C	C	E	E	H	H
B	BneM2	BneM2	Jejun2	Jejun2	Crop2	Crop2	Ovid2	Ovid2	Lung2	Lung2	Skin2	Skin2
C	Spleen2	Spleen2	Liver2	Liver2	Kidny2	Kidny2	Bursa2	Bursa2	Trach2	Trach2	Conj2	Conj2
D	Tngue2	Tngue2										
E	NTC	NTC	F	F	D	D	G	G	I	I	J	J
F	BneM2	BneM2	Jejun2	Jejun2	Crop2	Crop2	Ovid2	Ovid2	Lung2	Lung2	Skin2	Skin2
G	Spleen2	Spleen2	Liver2	Liver2	Kidny2	Kidny2	Bursa2	Bursa2	Trach2	Trach2	Conj2	Conj2
H	Tngue2	Tngue2										

Plate for G2 and 18S for chicken 1 (female)

E

	1	2	3	4	5	6	7	8	9	10	11	12
A	F	D	G	I	J	BneM1	Jejun1	Crop1	Testes1	Lung1	Skin1	Spleen1
B	Liver1	Kidny1	Bursa1	Trach1	Conj1	Tngue1	BneM2	Jejun2	Crop2	Ovid2	Lung2	Skin2
C	Spleen2	Liver2	Kidny2	Bursa2	Trach2	Conj2	Tngue2					
D												
E												
F												
G												
H												

NRC plate for 18S for both chickens – all samples in singlet, including standard curve and calibrator samples for 18S as well …

MASTER MIXES FOR THE FINAL PLATE SET-UPS:

One-Step Real-Time RT-PCR Set-Up

	One-Step MM	5695.24 uL		xtra ea.made	
32 uL prepared/Well	RT	284.76 uL	Set 1/sample	119.88 uL	
27 uL used/Well	Total MMRT prepared:	5980.00 uL	Set 1/MM ea	1942.06 uL	Duplicates
			Set 2/sample	0.00 uL	
	Total MMRT needed:	5511.24 uL	Set 2/MM ea	0.00 uL	Other

468.76 uL extra made

Well size prepared: 32.4 uL
RNA added/well: 8.424 uL

152 samples prepared
304 wells prepared

G-1

Fwd primer	262.44 uL	split	
Rev primer	262.44 uL	into	
Probe	39.37 uL	38	
MMRT:	1377.81 uL	47.95 uL	amounts
Water:	0.00 uL	then add	16.85 uL RNA to each

G-2

Fwd primer	262.44 uL	split	
Rev primer	262.44 uL	into	
Probe	39.37 uL	38	
MMRT:	1377.81 uL	47.95 uL	amounts
Water:	0.00 uL	then add	16.85 uL RNA to each

Ch18S

Fwd primer	524.88 uL	split	
Rev primer	524.88 uL	into	
Probe	78.73 uL	76	
MMRT:	2755.62 uL	47.95 uL	amounts
Water:	0.00 uL	then add	16.85 uL RNA to each

One-Step Real-Time RT-PCR NRC Set-Up

	One-Step MM	657.14 uL		xtra ea.made	
32 uL prepared/Well	WATER	32.86 uL	Set 1/sample	119.88 uL	
27 uL used/Well	Total MMW prepared:	690.00 uL	Set 1/MM ea	863.14 uL	Singlets
			Set 2/sample	0.00 uL	
	Total MMRT needed:	612.36 uL	Set 2/MM ea	0.00 uL	Other

77.64 uL extra made

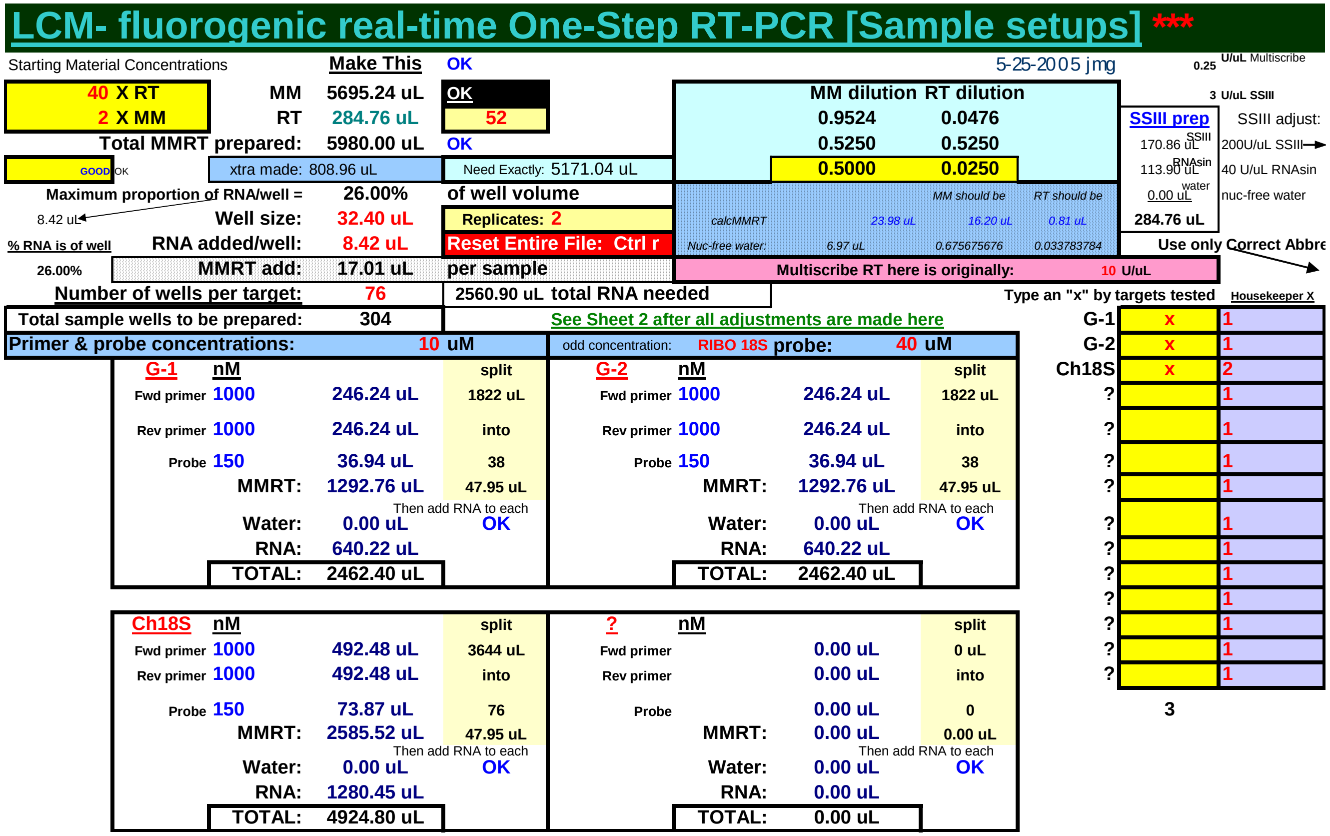
Well size prepared: 32.4 uL
RNA added/well: 8.424 uL

31 samples prepared
31 wells prepared

Ch18S

Fwd primer	116.64 uL	split	
Rev primer	116.64 uL	into	
Probe	17.50 uL	31	
MMW:	612.36 uL	23.98 uL	amounts
Water:	0.00 uL	then add	8.42 uL RNA to each

THE ADJUSTABLE PORTIONS OF THE MASTER-MIX CALCULATION/CREATION FILES ABOVE:



LCM- fluorogenic real-time One-Step RT-PCR [Sample setups] ***

Starting Material Concentrations | **Make This** | OK | 5-25-2005 jmg | 0.25 U/uL Multiscribe

3 U/uL SSIII

40 X RT	MM	5695.24 uL	OK	
2 X MM	RT	284.76 uL	52	
	Total MMRT prepared:	5980.00 uL	OK	
GOOD OK		xtra made: 808.96 uL	Need Exactly: 5171.04 uL	
Maximum proportion of RNA/well =		26.00%	of well volume	
8.42 uL	Well size:	32.40 uL	Replicates: 2	
% RNA is of well	RNA added/well:	8.42 uL	Reset Entire File: Ctrl r	
26.00%	MMRT add:	17.01 uL	per sample	
	Number of wells per target:	76	2560.90 uL total RNA needed	
	Total sample wells to be prepared:	304	See Sheet 2 after all adjustments are made here	

	MM dilution	RT dilution	
	0.9524	0.0476	
	0.5250	0.5250	
	0.5000	0.0250	
		MM should be	RT should be
calcMMRT	23.98 uL	16.20 uL	0.81 uL
Nuc-free water:	6.97 uL	0.675675676	0.033783784

SSIII prep	SSIII adjust:
170.86 uL SSIII	200U/uL SSIII→
113.90 uL RNAsin	40 U/uL RNAsin
0.00 uL water	nuc-free water
284.76 uL	

Use only Correct Abbre

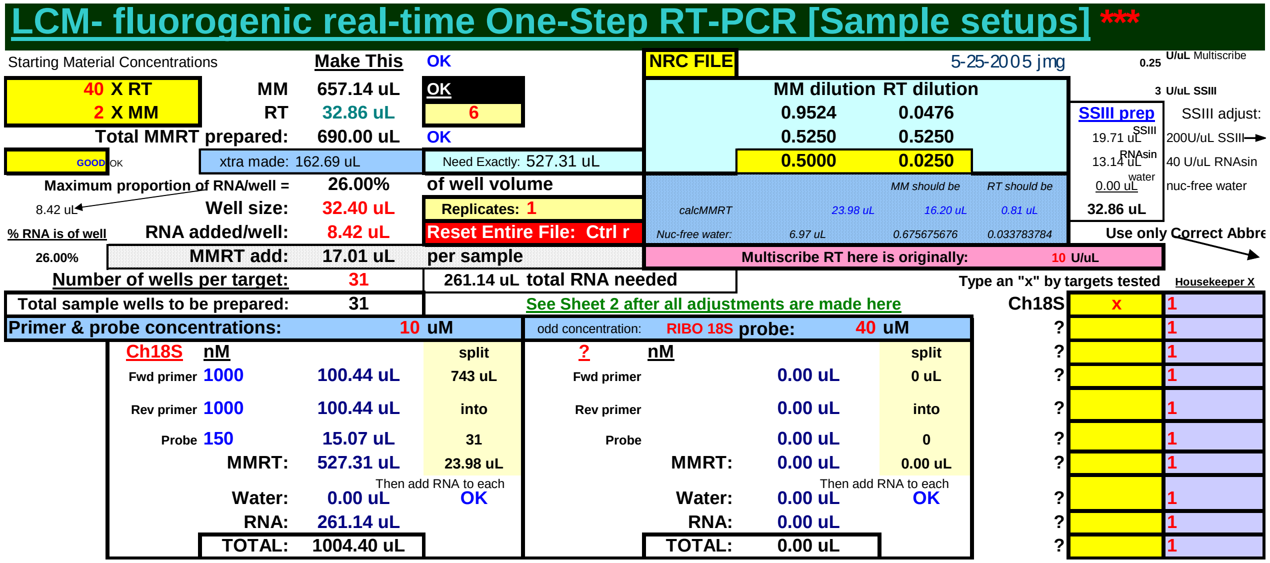
Multiscribe RT here is originally: 10 U/uL

Primer & probe concentrations: 10 uM | odd concentration: RIBO 18S probe: 40 uM

G-1	nM		split	G-2	nM		split
Fwd primer	1000	246.24 uL	1822 uL	Fwd primer	1000	246.24 uL	1822 uL
Rev primer	1000	246.24 uL	into	Rev primer	1000	246.24 uL	into
Probe	150	36.94 uL	38	Probe	150	36.94 uL	38
MMRT:		1292.76 uL	47.95 uL	MMRT:		1292.76 uL	47.95 uL
			Then add RNA to each				Then add RNA to each
Water:		0.00 uL	OK	Water:		0.00 uL	OK
RNA:		640.22 uL		RNA:		640.22 uL	
TOTAL:		2462.40 uL		TOTAL:		2462.40 uL	

Ch18S	nM		split	?	nM		split
Fwd primer	1000	492.48 uL	3644 uL	Fwd primer		0.00 uL	0 uL
Rev primer	1000	492.48 uL	into	Rev primer		0.00 uL	into
Probe	150	73.87 uL	76	Probe		0.00 uL	0
MMRT:		2585.52 uL	47.95 uL	MMRT:		0.00 uL	0.00 uL
			Then add RNA to each				Then add RNA to each
Water:		0.00 uL	OK	Water:		0.00 uL	OK
RNA:		1280.45 uL		RNA:		0.00 uL	
TOTAL:		4924.80 uL		TOTAL:		0.00 uL	

Type an "x" by targets tested		Housekeeper X
G-1	x	1
G-2	x	1
Ch18S	x	2
?		1
?		1
?		1
?		1
?		1
?		1
?		1
?		1
?		1
?		1
?		1
	3	



LCM- fluorogenic real-time One-Step RT-PCR [Sample setups] ***

Starting Material Concentrations | **Make This** | OK | NRC FILE | 5-25-2005 jmg | 0.25 U/uL Multiscribe

3 U/uL SSIII

40 X RT	MM	657.14 uL	OK	
2 X MM	RT	32.86 uL	6	
	Total MMRT prepared:	690.00 uL	OK	
GOOD OK		xtra made: 162.69 uL	Need Exactly: 527.31 uL	
Maximum proportion of RNA/well =		26.00%	of well volume	
8.42 uL	Well size:	32.40 uL	Replicates: 1	
% RNA is of well	RNA added/well:	8.42 uL	Reset Entire File: Ctrl r	
26.00%	MMRT add:	17.01 uL	per sample	
	Number of wells per target:	31	261.14 uL total RNA needed	
	Total sample wells to be prepared:	31	See Sheet 2 after all adjustments are made here	

	MM dilution	RT dilution	
	0.9524	0.0476	
	0.5250	0.5250	
	0.5000	0.0250	
		MM should be	RT should be
calcMMRT	23.98 uL	16.20 uL	0.81 uL
Nuc-free water:	6.97 uL	0.675675676	0.033783784

SSIII prep	SSIII adjust:
19.71 uL SSIII	200U/uL SSIII→
13.14 uL RNAsin	40 U/uL RNAsin
0.00 uL water	nuc-free water
32.86 uL	

Use only Correct Abbre

Multiscribe RT here is originally: 10 U/uL

Primer & probe concentrations: 10 uM | odd concentration: RIBO 18S probe: 40 uM

Ch18S	nM		split	?	nM		split
Fwd primer	1000	100.44 uL	743 uL	Fwd primer		0.00 uL	0 uL
Rev primer	1000	100.44 uL	into	Rev primer		0.00 uL	into
Probe	150	15.07 uL	31	Probe		0.00 uL	0
MMRT:		527.31 uL	23.98 uL	MMRT:		0.00 uL	0.00 uL
			Then add RNA to each				Then add RNA to each
Water:		0.00 uL	OK	Water:		0.00 uL	OK
RNA:		261.14 uL		RNA:		0.00 uL	
TOTAL:		1004.40 uL		TOTAL:		0.00 uL	

Type an "x" by targets tested		Housekeeper X
Ch18S	x	1
?		1
?		1
?		1
?		1
?		1
?		1
?		1
?		1
?		1

~DATA PROCESSING AND RESULTS~

PARTIAL DEPICTION OF qPCR DATA CRUNCHING FILE – THE FORMAT USED FOR EACH PLATE RESULTS:

Well	Type	Name	Primer/Probe	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty
A1	NTC	G1	G1	50	0			
A2	NTC	G1	G1	50	0		avg'd Cts	
A3	UNKN	CALB	G1	27.84	0.15	1.84E-01	27.74	
A4	UNKN	CALB	G1	27.63	0.15	2.12E-01		
A5	STND		G1	25.53	0	1	25.53	
A6	STND		G1	25.53	0	1		
A7	STND		G1	28.53	0	1.00E-01	28.51	
A8	STND		G1	28.48	0	1.00E-01		
A9	STND		G1	32.07	0	1.00E-02	32.06	
A10	STND		G1	32.04	0	1.00E-02		
A11	STND		G1	35.76	0	1.00E-03	35.66	
A12	STND		G1	35.56	0	1.00E-03		
B1	UNKN	1	G1	24.19	2.98	2.2	24.21	
B2	UNKN	1	G1	24.23	2.98	2.14		
B3	UNKN	2	G1	35.33	9.61	1.14E-03	35.23	
B4	UNKN	2	G1	35.13	9.61	1.31E-03		
B5	UNKN	3	G1	36.59	10.29	4.87E-04	36.60	
B6	UNKN	3	G1	36.6	10.29	4.83E-04		
B7	UNKN	4	G1	32.12	7.43	1.01E-02	32.18	
B8	UNKN	4	G1	32.23	7.43	9.34E-03		
B9	UNKN	5	G1	30.66	6.73	2.72E-02	30.67	
B10	UNKN	5	G1	30.68	6.73	2.69E-02		
B11	UNKN	6	G1	37.61	10.58	2.44E-04	37.38	
B12	UNKN	6	G1	37.14	10.58	3.36E-04		
C1	UNKN	7	G1	35.29	9.66	1.18E-03	35.32	
C2	UNKN	7	G1	35.35	9.66	1.13E-03		
C3	UNKN	8	G1	33.28	8.03	4.61E-03	33.28	
C4	UNKN	8	G1	33.28	8.03	4.60E-03		
C5	UNKN	9	G1	30.31	6.44	3.44E-02	30.26	
C6	UNKN	9	G1	30.21	6.44	3.68E-02		
C7	UNKN	10	G1	33.42	8.6	4.18E-03	33.57	
C8	UNKN	10	G1	33.72	8.6	3.41E-03		
C9	UNKN	11	G1	38.14	10.63	1.70E-04	38.02	
C10	UNKN	11	G1	37.9	10.63	2.00E-04		
C11	UNKN	12	G1	34.3	8.76	2.30E-03	34.20	
C12	UNKN	12	G1	34.09	8.76	2.66E-03		
D1	UNKN	13	G1	35.85	7.08	8.06E-04	36.03	
D2	UNKN	13	G1	36.2	7.08	6.35E-04		
E1	NTC	18S	G18S	33.04	5.24		36.74	
E2	NTC	18S	G18S	40.44	5.24			
E3	UNKN	CALF	G18S	17.35	0.05	3.64E-01	17.32	
E4	UNKN	CALF	G18S	17.28	0.05	3.80E-01		
E5	STND		G18S	16.03	0	1	16.04	
E6	STND		G18S	16.04	0	1		
E7	STND		G18S	18.1	0	2.00E-01	18.09	
E8	STND		G18S	18.08	0	2.00E-01		
E9	STND		G18S	20.3	0	4.00E-02	20.32	
E10	STND		G18S	20.33	0	4.00E-02		
E11	STND		G18S	23.16	0	8.00E-03	23.16	
E12	STND		G18S	23.16	0	8.00E-03		
F1	UNKN	1	G18S	19.04	2.98	1.14E-01	19.05	
F2	UNKN	1	G18S	19.05	2.98	1.14E-01		
F3	UNKN	2	G18S	18.72	9.61	1.43E-01	18.60	
F4	UNKN	2	G18S	18.48	9.61	1.68E-01		
F5	UNKN	3	G18S	18.78	10.29	1.37E-01	18.77	
F6	UNKN	3	G18S	18.76	10.29	1.39E-01		
F7	UNKN	4	G18S	19.32	7.43	9.49E-02	19.31	
F8	UNKN	4	G18S	19.29	7.43	9.66E-02		
F9	UNKN	5	G18S	19.07	6.73	1.12E-01	19.02	
F10	UNKN	5	G18S	18.97	6.73	1.21E-01		
F11	UNKN	6	G18S	19.07	10.58	1.12E-01	19.05	
F12	UNKN	6	G18S	19.02	10.58	1.16E-01		
G1	UNKN	7	G18S	18.58	9.66	1.56E-01	18.59	
G2	UNKN	7	G18S	18.59	9.66	1.56E-01		
G3	UNKN	8	G18S	19.39	8.03	9.03E-02	19.36	
G4	UNKN	8	G18S	19.33	8.03	9.38E-02		
G5	UNKN	9	G18S	19.1	6.44	1.10E-01	19.11	
G6	UNKN	9	G18S	19.11	6.44	1.09E-01		
G7	UNKN	10	G18S	18.63	8.6	1.52E-01	18.67	
G8	UNKN	10	G18S	18.71	8.6	1.43E-01		
G9	UNKN	11	G18S	19.64	10.63	7.61E-02	19.61	
G10	UNKN	11	G18S	19.58	10.63	7.93E-02		
G11	UNKN	12	G18S	19.09	8.76	1.11E-01	19.02	

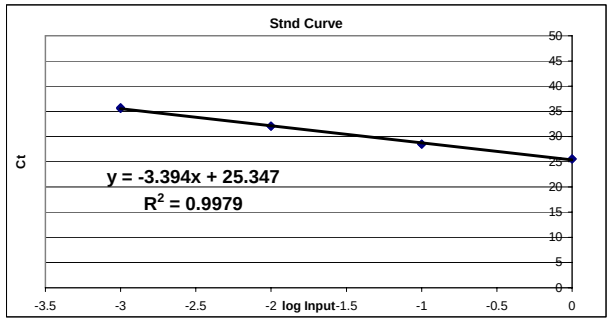
G1

Stnd curve

log input	Ct
0	25.53
0	25.53
-1	28.53
-1	28.48
-2	32.07
-2	32.04
-3	35.76
-3	35.56

m = -3.394
b = 25.3465

97.08% Efficiency of Reaction
-0.998929 Correlation



G18S

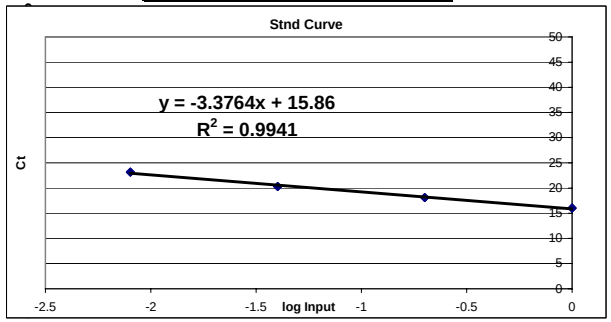
Stnd curve

log input	Ct
0	16.03
0	16.04
-0.69897	18.1
-0.69897	18.08
-1.39794	20.3
-1.39794	20.33
-2.09691	23.16
-2.09691	23.16

m = -3.376397
b = 15.86

97.78% Efficiency of Reaction
-0.997024 Correlation

NRC
37.5
37.5
38.6
38.6
37.09
37.09
36.85
36.85
37.84
37.84
38.74
38.74
37.37
37.37
37.35
37.35
37.22
37.22
37.71
37.71
37.9
37.9
38.41



0
0
0
0
0
0

DATA CRUNCHING FILE (CONT.)

G1
Qty Calculations:

NTC	0	
NTC	0	
CALB	0.184213373	
CALB	0.212419827	
1	2.191534841	BoneM1
1	2.132862584	BoneM1
2	0.001144157	Jej1
2	0.001310428	Jej1
3	0.000486679	Crop1
3	0.000483388	Crop1
4	0.010098858	Testes1
4	0.009372644	Testes1
5	0.027192124	Lung1
5	0.026825658	Lung1
6	0.000243619	Skin1
6	0.000335113	Skin1
7	0.001175631	Spleen1
7	0.001128737	Spleen1
8	0.004597192	Liver1
8	0.004597192	Liver1
9	0.034479923	Kidney1
9	0.036900313	Kidney1
10	0.004180646	Bursa1
10	0.003410767	Bursa1
11	0.000170041	Trach1
11	0.000200109	Trach1
12	0.002301242	Conj1
12	0.002653604	Conj1
13	0.000804033	Tongue1
13	0.00063409	Tongue1

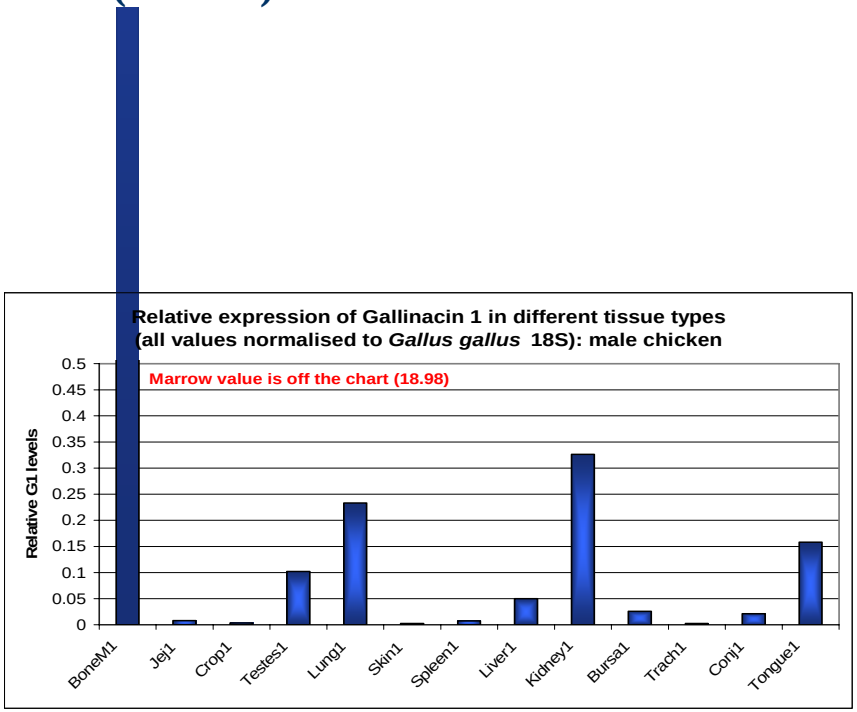
G18S
Qty Calculations:

NTC	8.16085E-06	
NTC	5.24868E-08	
CALF	0.361993808	
CALF	0.379693622	
1	0.11433193	BoneM1
1	0.113554879	BoneM1
2	0.142214255	Jej1
2	0.167503862	Jej1
3	0.13651259	Crop1
3	0.138387282	Crop1
4	0.094458032	Testes1
4	0.096410449	Testes1
5	0.112016584	Lung1
5	0.119922231	Lung1
6	0.112016584	Skin1
6	0.115902022	Skin1
7	0.156461487	Spleen1
7	0.155398104	Spleen1
8	0.090054772	Liver1
8	0.093816052	Liver1
9	0.109748126	Kidney1
9	0.109002228	Kidney1
10	0.151216355	Bursa1
10	0.143187421	Bursa1
11	0.075938724	Trach1
11	0.079110426	Trach1
12	0.110499128	Conj1
12	0.122400984	Conj1
13	0.00424276	Tongue1
13	0.004896039	Tongue1

Target Normalised to Housekeeper:

BoneM1	19.23376
BoneM1	18.71883
Jej1	0.007438
Jej1	0.008519
Crop1	0.003541
Crop1	0.003517
Testes1	0.105831
Testes1	0.098221
Lung1	0.234749
Lung1	0.231586
Skin1	0.002138
Skin1	0.002941
Spleen1	0.00754
Spleen1	0.007239
Liver1	0.050026
Liver1	0.050026
Kidney1	0.315248
Kidney1	0.337378
Bursa1	0.028422
Bursa1	0.023188
Trach1	0.002194
Trach1	0.002582
Conj1	0.019813
Conj1	0.022847
Tongue1	0.176864
Tongue1	0.139482

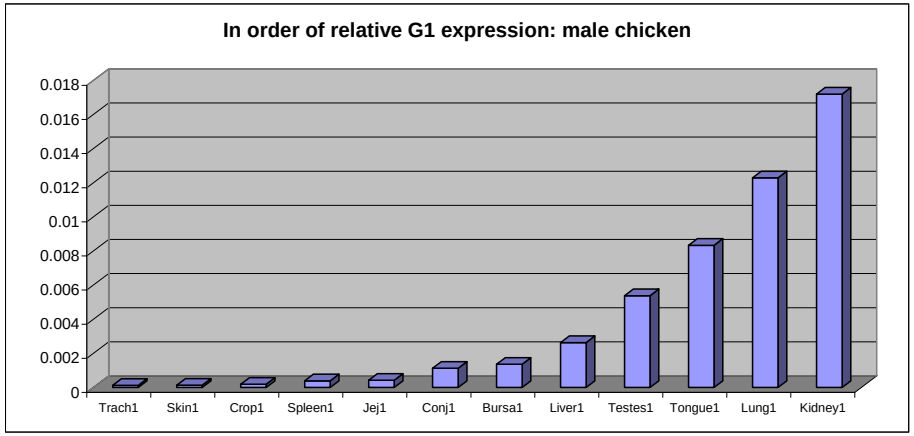
Averaged together	
BoneM1	18.9763
Jej1	0.007978
Crop1	0.003529
Testes1	0.102026
Lung1	0.233168
Skin1	0.00254
Spleen1	0.007389
Liver1	0.050026
Kidney1	0.326313
Bursa1	0.025805
Trach1	0.002388
Conj1	0.02133
Tongue1	0.158173



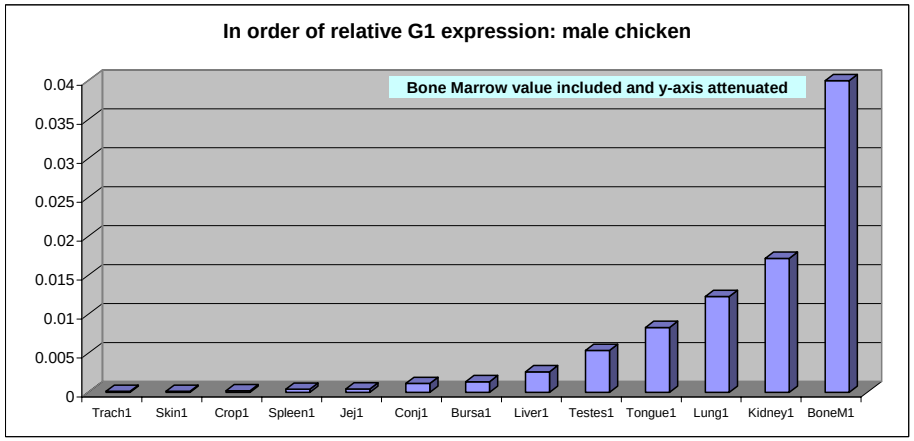
DATA CRUNCHING FILE (CONT.)

Sorted:
Ctrl s

Trach1	0.000126
Skin1	0.000134
Crop1	0.000186
Spleen1	0.000389
Jej1	0.00042
Conj1	0.001124
Bursa1	0.00136
Liver1	0.002636
Testes1	0.005376
Tongue1	0.008335
Lung1	0.012287
Kidney1	0.017196



Trach1	0.000126
Skin1	0.000134
Crop1	0.000186
Spleen1	0.000389
Jej1	0.00042
Conj1	0.001124
Bursa1	0.00136
Liver1	0.002636
Testes1	0.005376
Tongue1	0.008335
Lung1	0.012287
Kidney1	0.017196
BoneM1	1



Note: extramedullary hematopoiesis in Kidney possible ...
and, potential inflammation may exacerbate defensin expression

AMPLIFICATION PLOT RESUTLS: TARGET PLATES 1, 2, 3 AND 4

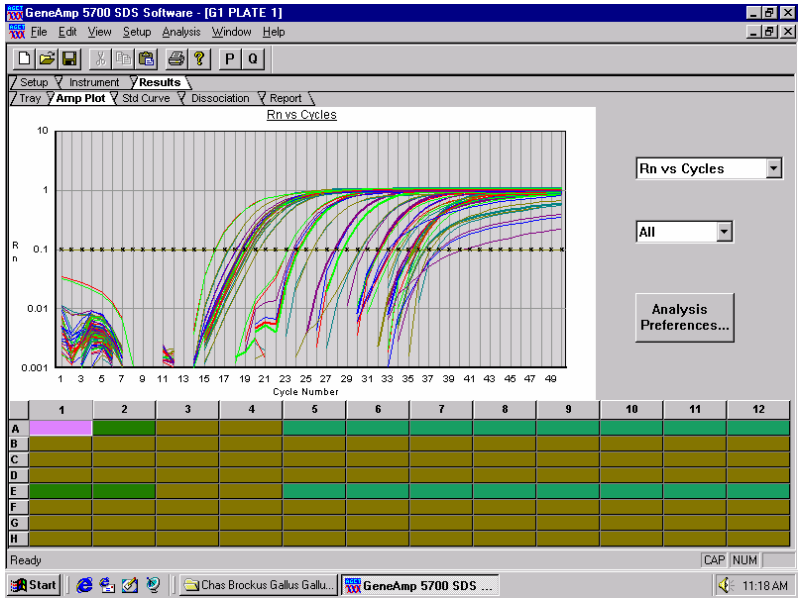


Plate 1 (G1 – male chicken)

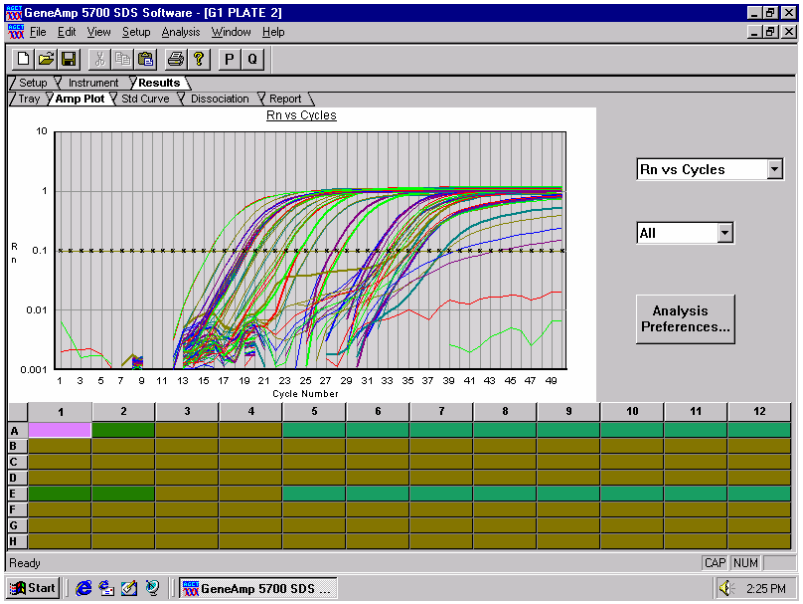


Plate 2 (G1 – female chicken)

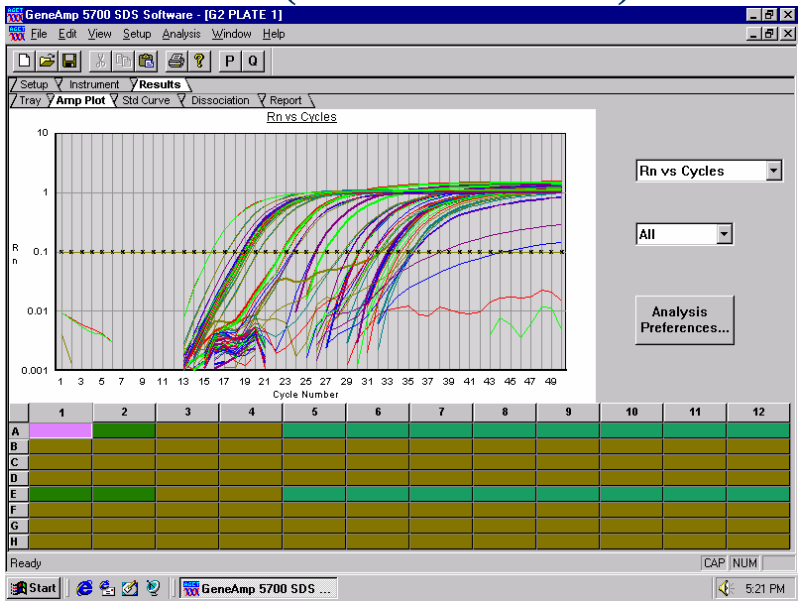


Plate 3 (G2 – male chicken)

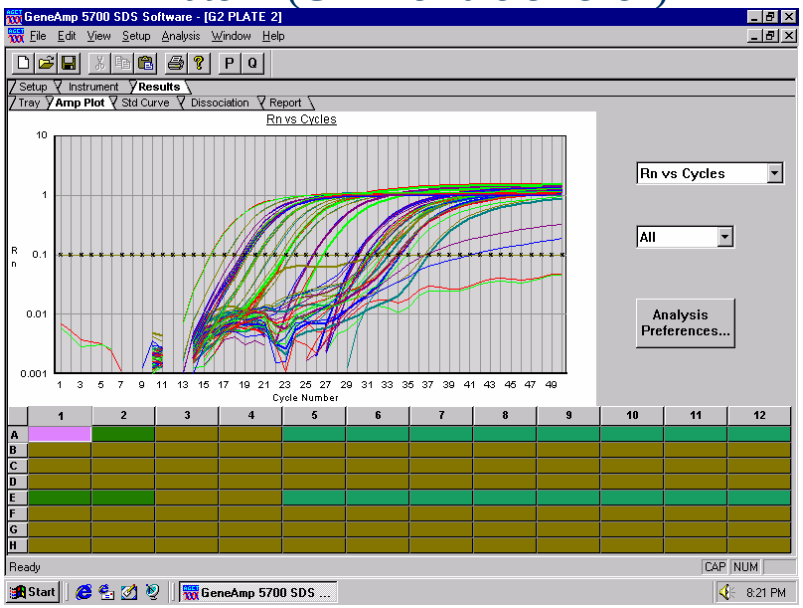
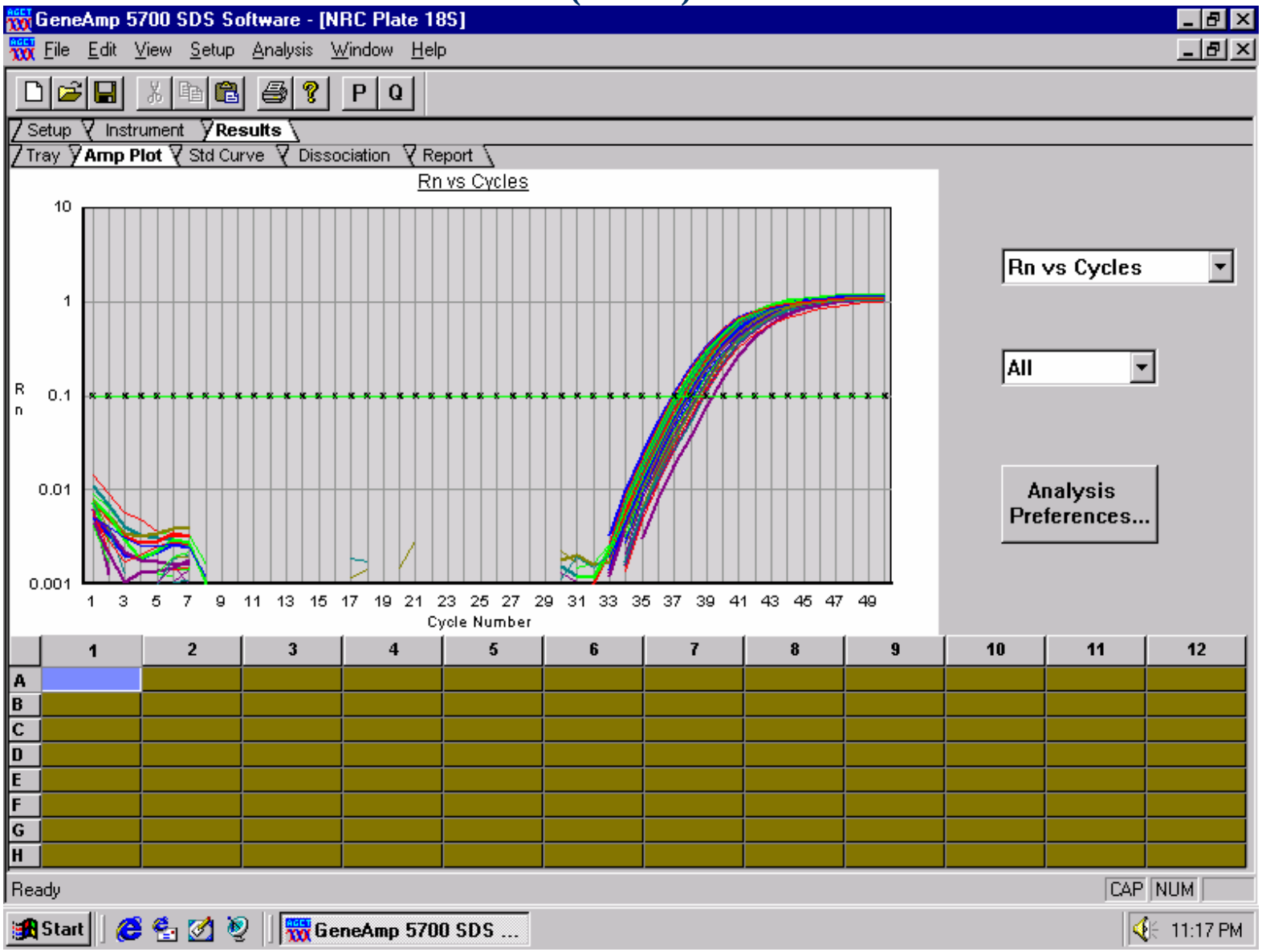


Plate 4 (G2 – female chicken)

NRC PLATE RESULTS:
(Plate 5)



NRC RESULTS SHOWED THAT GENOMIC DNA CONTAMINATION FOR EACH SAMPLE WAS MINIMAL AND IT DID NOT HAVE ANY IMPACT ON OUR FINAL QUANTITATIVE RESULTS.

Ct correction file for signals generated by genomic DNA contamination during qPCR as revealed by NRC plates:

Ct correction file for genomic DNA contamination signals generated during qPCR as revealed by NRC plates

File for real-time Pfaffl Math

Real-Time	Stnd Curve
Target m	-3.321928095
Target b	23
Efficiency:	100.00%
UnkwnCts	Rel. Qty
22	2
24.3	0.406126198
26	0.125
25	0.25
34	0.000488281

Real-Time	Stnd Curve
Housekeeper m	-3.351928095
Housekeeper b	16
Efficiency:	98.76%
UnkwnCts	Rel. Qty
21	0.032234521
18.5	0.179539748
22	0.016217558
21.33	0.02569629
31	3.34937E-05

The perfect slope:
-3.321928095

Results:
Targets normalized to Housekeepers

1	62.04528336
2	2.262040592
3	7.707695427
4	9.729030941
5	14.57828303

Frequencies expected between serial dilutions with the following serial dilution factors at different efficiencies

dilution series	Ideal frequency	Affected by Efficiency	Efficiency	Average Cts away from ideal frequency
1: 50	5.6439	6.2394	80.00%	0.5955
1: 40	5.3219	5.9174	80.00%	0.5955
1: 30	4.9069	5.5024	80.00%	0.5955
1: 25	4.6439	5.2394	80.00%	0.5955
1: 20	4.3219	4.9174	80.00%	0.5955
1: 15	3.9069	4.5024	80.00%	0.5955
1: 10	3.3219	3.9174	80.00%	0.5955
1: 9	3.1699	3.7654	80.00%	0.5955
1: 8	3.0000	3.5955	80.00%	0.5955
1: 7	2.8074	3.4029	80.00%	0.5955
1: 5	2.3219	2.9174	80.00%	0.5955
1: 3	1.5850	2.1805	80.00%	0.5955
1: 2	1.0000	1.5955	80.00%	0.5955

Worried about contaminating signals several Cts away from actual sample (target or housekeeper) signals?

10 average Cts away from Genuine Signal

Genuine RNA Target Signal		Contaminating DNA Target Signal		OR:
Real-Time	Stnd Curve	Real-Time	Stnd Curve	
Target m	-3.321928095	Target m	-3.321928095	
Target b	23	Target b	23	
Efficiency:	100.00%	Efficiency:	100.00%	Sample specific DNA
UnkwnCts	Rel. Qty	UnkwnCts	Rel. Qty	ΔCt from RNA signal
22	1.999511719	34	0.000488281	12
24.3	0.404539768	32.3	0.00158643	8
26	0.124999985	49	1.49012E-08	23
25	0.248046875	32	0.001953125	7
34	0.000487328	43	9.53674E-07	9

4.715 average Cts away from Genuine Signal

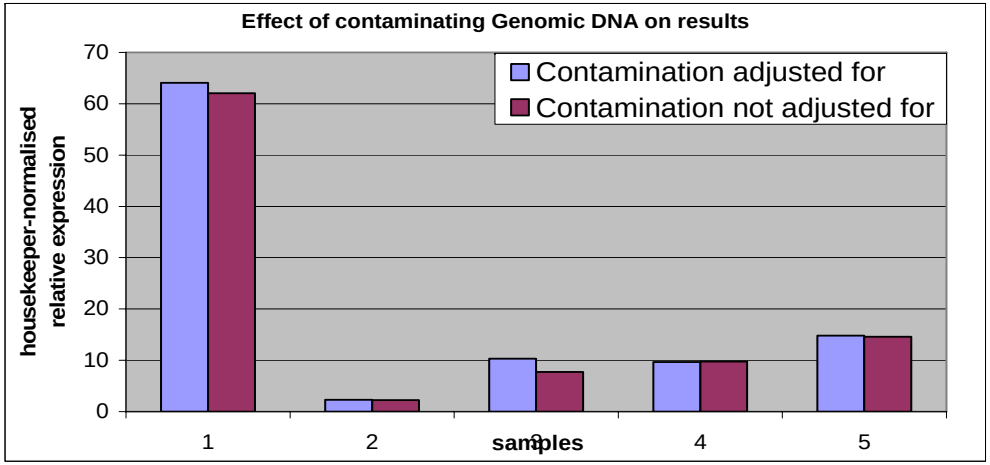
Genuine RNA Housekeeper Signal		Contaminating DNA Housekeeper Signal		OR:
Real-Time	Stnd Curve	Real-Time	Stnd Curve	
Housekeeper m	-3.351928095	Housekeeper m	-3.351928095	
Housekeeper b	16	Housekeeper b	16	
Efficiency:	98.76%	Efficiency:	98.76%	Sample specific DNA
UnkwnCts	Rel. Qty	UnkwnCts	Rel. Qty	ΔCt from RNA signal
21	0.031195457	26	0.001039064	5
18.5	0.17807484	25.5	0.001464908	7
22	0.012112551	24	0.004105008	2
21.33	0.025689532	33.33	6.75836E-06	12
31	3.29506E-05	37	5.43187E-07	6

Targets normalized to Housekeepers

				So, original Qty values need to be adjusted to
1	64.09624743	3.31%	from a perfect 100% uncontaminated result	103.31%
2	2.271740169	0.43%	from a perfect 100% uncontaminated result	100.43%
3	10.31987294	33.89%	from a perfect 100% uncontaminated result	133.89%
4	9.655562389	-0.76%	from a perfect 100% uncontaminated result	99.24%
5	14.78966203	1.45%	from a perfect 100% uncontaminated result	101.45%

of what they first appeared to be …

	Contamination adjusted for	Contamination not adjusted for
1	64.09624743	62.04528336
2	2.271740169	2.262040592
3	10.31987294	7.707695427
4	9.655562389	9.729030941
5	14.78966203	14.57828303



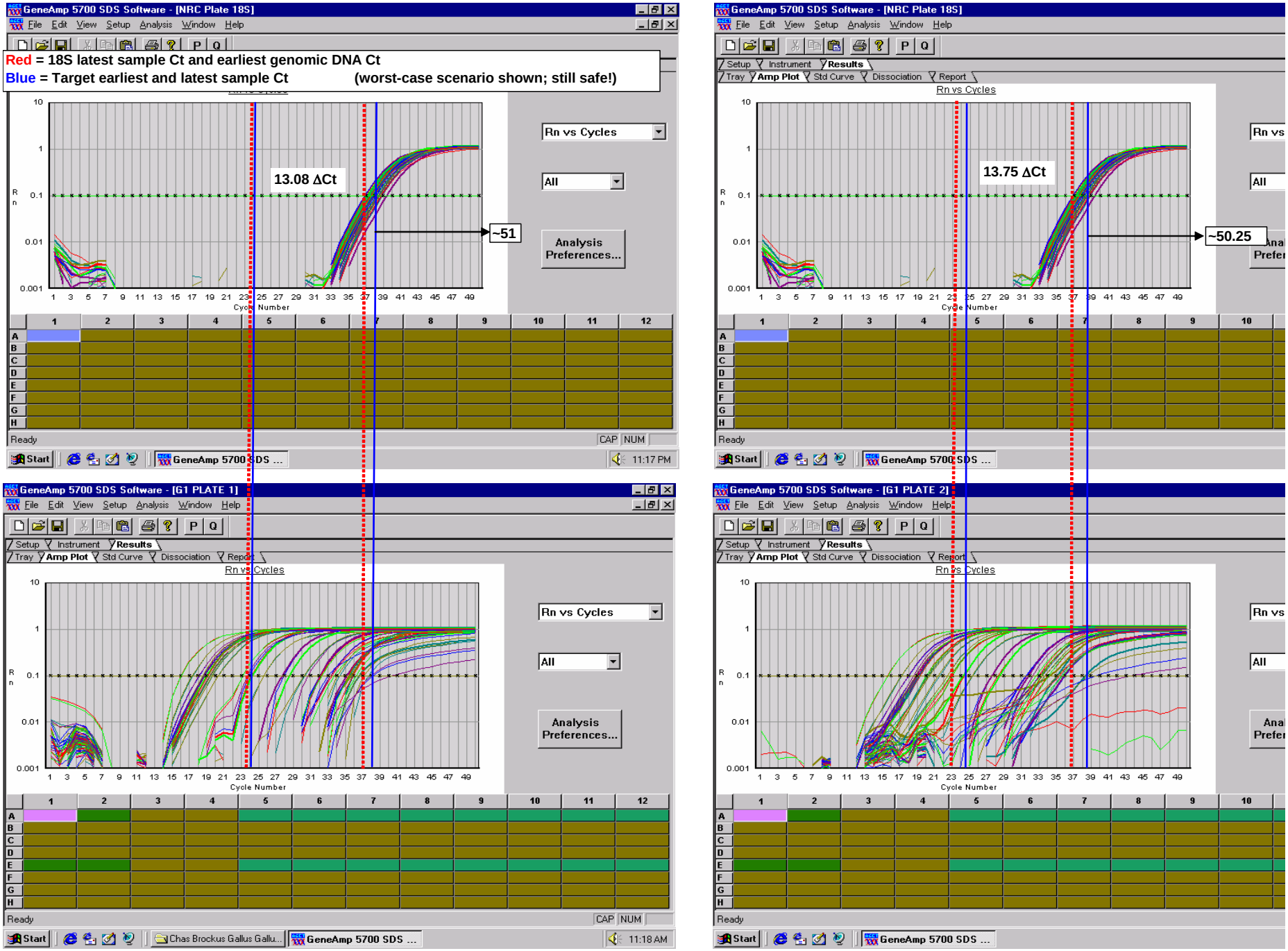
CALCULATION RESULTS PROVING THE ABOVE POINT REGARDING GENOMIC DNA:

Cts away from genuine signal that contaminating signal appears or is predicted to appear - based on dilution factors and amp eff. of 18S

	G1 predicted	G2	18S	
B	17.91	19.85	20.02	F
A	17.96	19.77	21.07	D
C	17.57	19.38	19.79	G
E	17.01	18.89	19.02	I
H	15.82	18.66	14.02	J
	18.46	18.49	18.47	
	20.00	19.96	19.98	
	18.32	18.30	18.31	
	17.55	17.64	17.59	
	18.82	18.86	18.84	
	19.70	19.73	19.71	
	18.79	18.82	18.80	
	17.99	18.06	18.03	
	18.12	18.07	18.09	
	19.04	19.08	19.06	
	18.29	18.38	18.34	
	19.40	19.42	19.41	
	14.42	14.56	14.49	
	19.39	19.45	19.42	
	16.71	16.53	16.62	
	15.24	14.99	15.12	
	19.05	19.02	19.04	
	14.89	14.89	14.89	
	19.19	19.15	19.17	
	18.94	18.85	18.90	
	18.73	18.68	18.70	
	17.91	17.72	17.81	
	19.83	19.69	19.76	
	16.93	16.92	16.92	
	18.82	18.79	18.81	
	17.81	17.84	17.82	

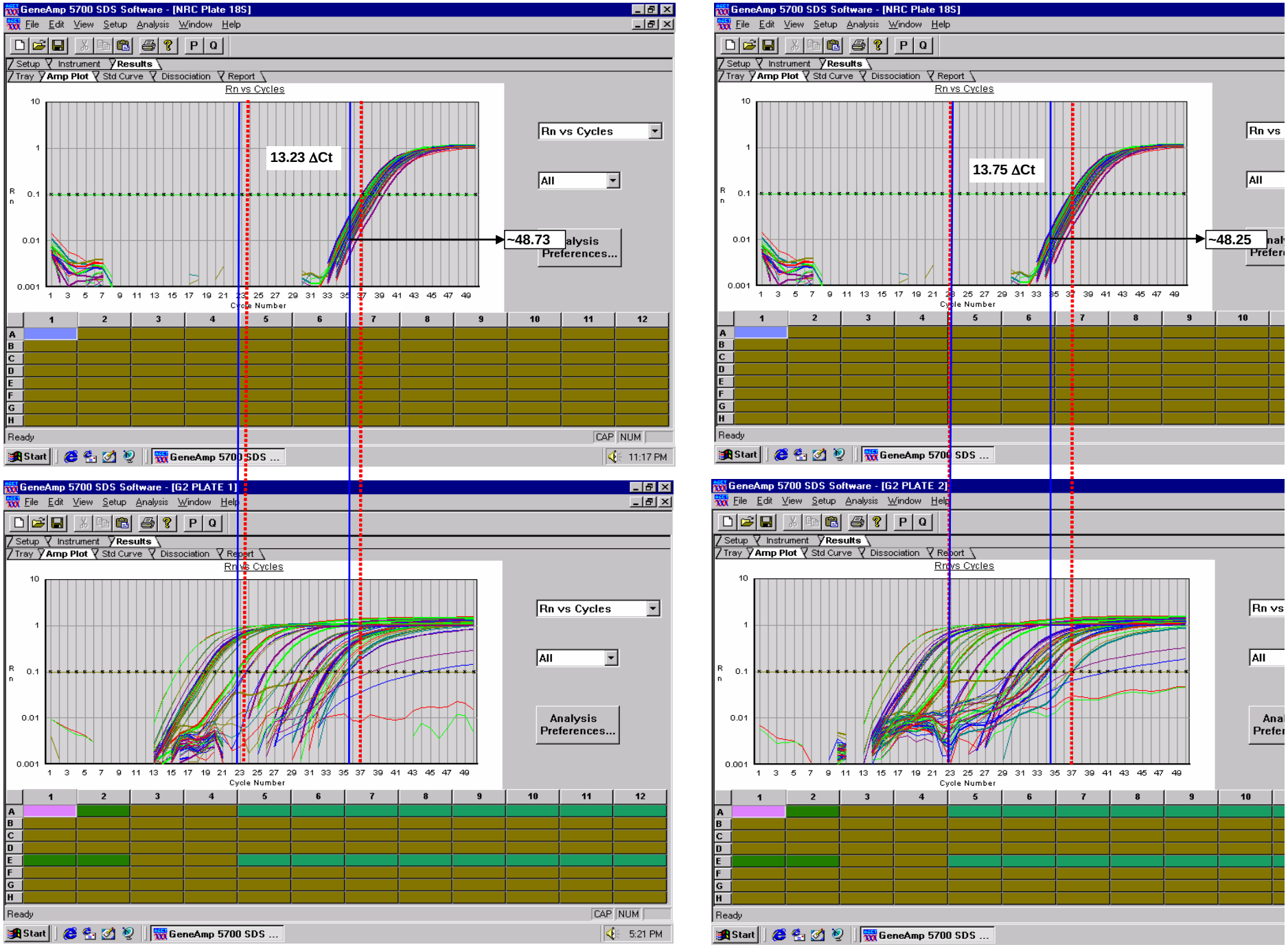
ANOTHER WAY WE USE TO VISUALIZE THE INABILITY OF GENOMIC DNA CONTAMINATION TO AFFECT OUR REAL-TIME qPCR QUANTITATION CALCULATIONS:

WORST CASE SCENARIO SHOWN – AND STILL, WE ARE SAFE …



Contaminating DNA signals more than 8 cycles away from genuine mRNA signals have no affect on quantitation. Here, all contaminating signals are >13 cycles away from their respective mRNA signals …

THE ABOVE PROCESS CONTINUED … (REGARDING GENOMIC DNA)



Contaminating DNA signals more than 8 cycles away from genuine mRNA signals have no affect on quantitation. Here, all contaminating signals are >13 cycles away from their respective mRNA signals …

MATHEMATICAL PROOF SHOWING HOW A CONTAMINATING GENOMIC DNA REAL-TIME qPCR SIGNALS AT ONLY 8 CYCLES AWAY FROM GENUINE mRNA REAL-TIME qPCR SIGNALS HAVE ALMOST NO EFFECT ON RESULTS:

Ct correction file for genomic DNA contamination signals generated during qPCR as revealed by NRC plates

File for real-time Pfaffl Math

Real-Time	Stnd Curve
Target m	-3.391928095
Target b	23
Efficiency:	97.16%
UnkwnCts	Rel. Qty
22	1.971594375
24.3	0.413749192
26	0.130481004
25	0.257255613
34	0.000571487

Real-Time	Stnd Curve
Housekeeper m	-3.351928095
Housekeeper b	16
Efficiency:	98.76%
UnkwnCts	Rel. Qty
21	0.032234521
18.5	0.179539748
22	0.016217558
21.33	0.02569629
31	3.34937E-05

The perfect slope:
-3.321928095

Results:
Targets normalized to Housekeepers

1	61.16406583
2	2.304499121
3	8.045662683
4	10.01139127
5	17.06251536

Frequencies expected between serial dilutions with the following serial dilution factors at different efficien

dilution series	Ideal frequency	Affected by Efficiency	Efficiency	Average C from ideal f
1: 50	5.6439	6.2394	80.00%	0.5955
1: 40	5.3219	5.9174	80.00%	0.5955
1: 30	4.9069	5.5024	80.00%	0.5955
1: 25	4.6439	5.2394	80.00%	0.5955
1: 20	4.3219	4.9174	80.00%	0.5955
1: 15	3.9069	4.5024	80.00%	0.5955
1: 10	3.3219	3.9174	80.00%	0.5955
1: 9	3.1699	3.7654	80.00%	0.5955
1: 8	3.0000	3.5955	80.00%	0.5955
1: 7	2.8074	3.4029	80.00%	0.5955
1: 5	2.3219	2.9174	80.00%	0.5955
1: 3	1.5850	2.1805	80.00%	0.5955
1: 2	1.0000	1.5955	80.00%	0.5955

Worried about contaminating signals several Cts away from actual sample (target or housekeeper) signals?

8 average Cts away from Genuine Signal

Genuine RNA Target Signal

Real-Time	Stnd Curve
Target m	-3.391928095
Target b	23
Efficiency:	97.16%
UnkwnCts	Rel. Qty
22	1.962959083
24.3	0.411937032
26	0.129909516
25	0.256128871
34	0.000568984

Contaminating DNA Target Signal

Real-Time	Stnd Curve	OR: Sample specific DNA ΔCt from RNA signal
Target m	-3.391928095	
Target b	23	
Efficiency:	97.16%	
UnkwnCts	Rel. Qty	
30	0.008635292	0
32.3	0.00181216	0
34	0.000571487	0
33	0.001126742	0
42	2.50303E-06	0

8 average Cts away from Genuine Signal

Genuine RNA Housekeeper Signal

Real-Time	Stnd Curve
Housekeeper m	-3.351928095
Housekeeper b	16
Efficiency:	98.76%
UnkwnCts	Rel. Qty
21	0.032102198
18.5	0.178802736
22	0.016150985
21.33	0.025590806
31	3.33562E-05

Contaminating DNA Housekeeper Signal

Real-Time	Stnd Curve	OR: Sample specific DNA ΔCt from RNA signal
Housekeeper m	-3.351928095	
Housekeeper b	16	
Efficiency:	98.76%	
UnkwnCts	Rel. Qty	
29	0.000132323	0
26.5	0.000737012	0
30	6.65732E-05	0
29.33	0.000105483	0
39	1.37492E-07	0

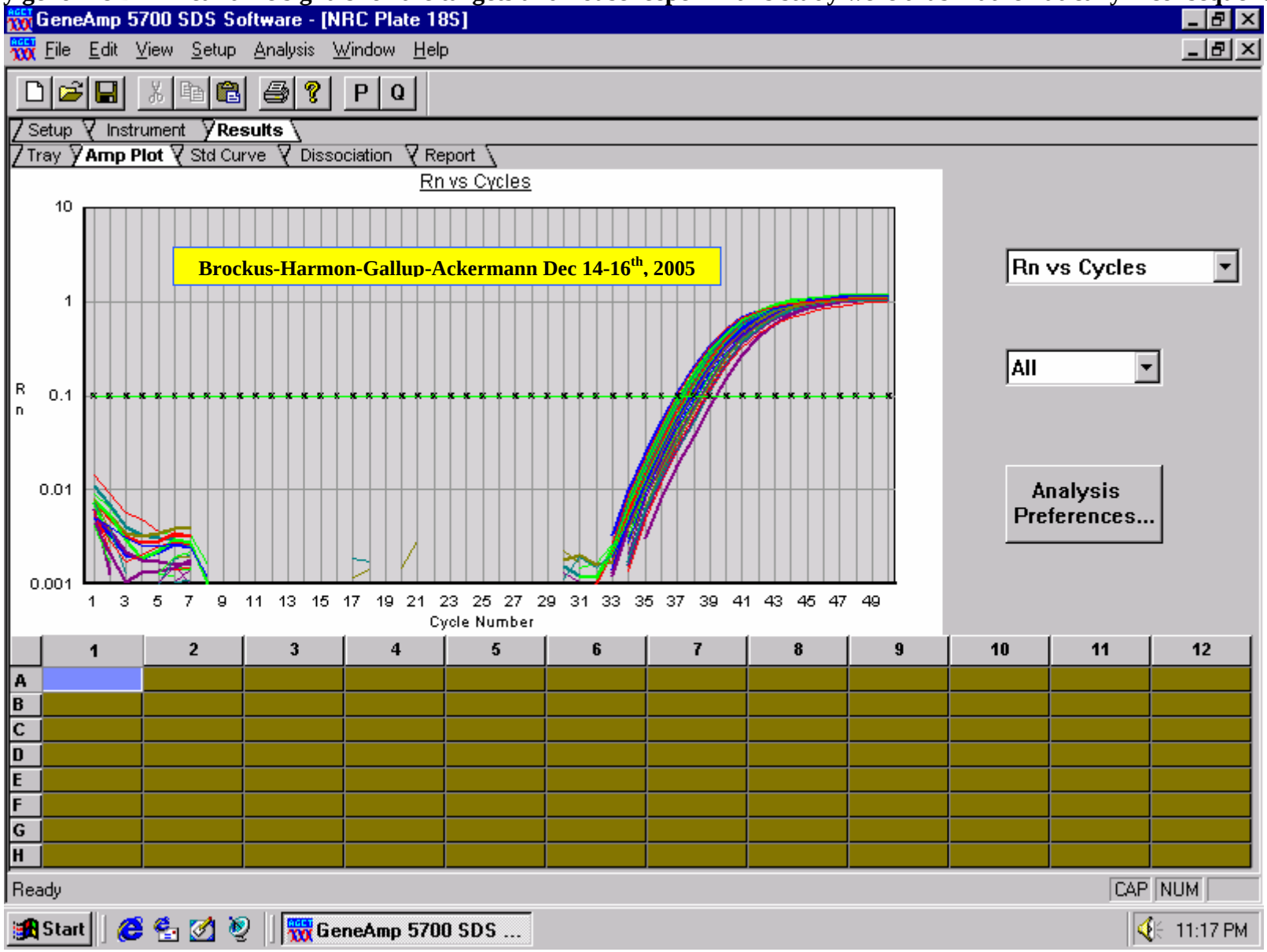
Targets normalized to Housekeepers

			So, original Qty values need to be adjusted to
1	61.14718593	-0.03% from a perfect 100% uncontaminated result	99.97%
2	2.303863131	-0.03% from a perfect 100% uncontaminated result	99.97% of what they first appeared t
3	8.043442262	-0.03% from a perfect 100% uncontaminated result	99.97%
4	10.00862835	-0.03% from a perfect 100% uncontaminated result	99.97%
5	17.05780649	-0.03% from a perfect 100% uncontaminated result	99.97%

NOTICE THAT GENOMIC DNA CONTAMINATION APPEARING AS EARLY AS 8 CYCLES AWAY FROM GENUINE TARGET SIGNALS ONLY ALTERS THE FINAL QUANTITATION VALUES BY 0.03% …!

So … once again:

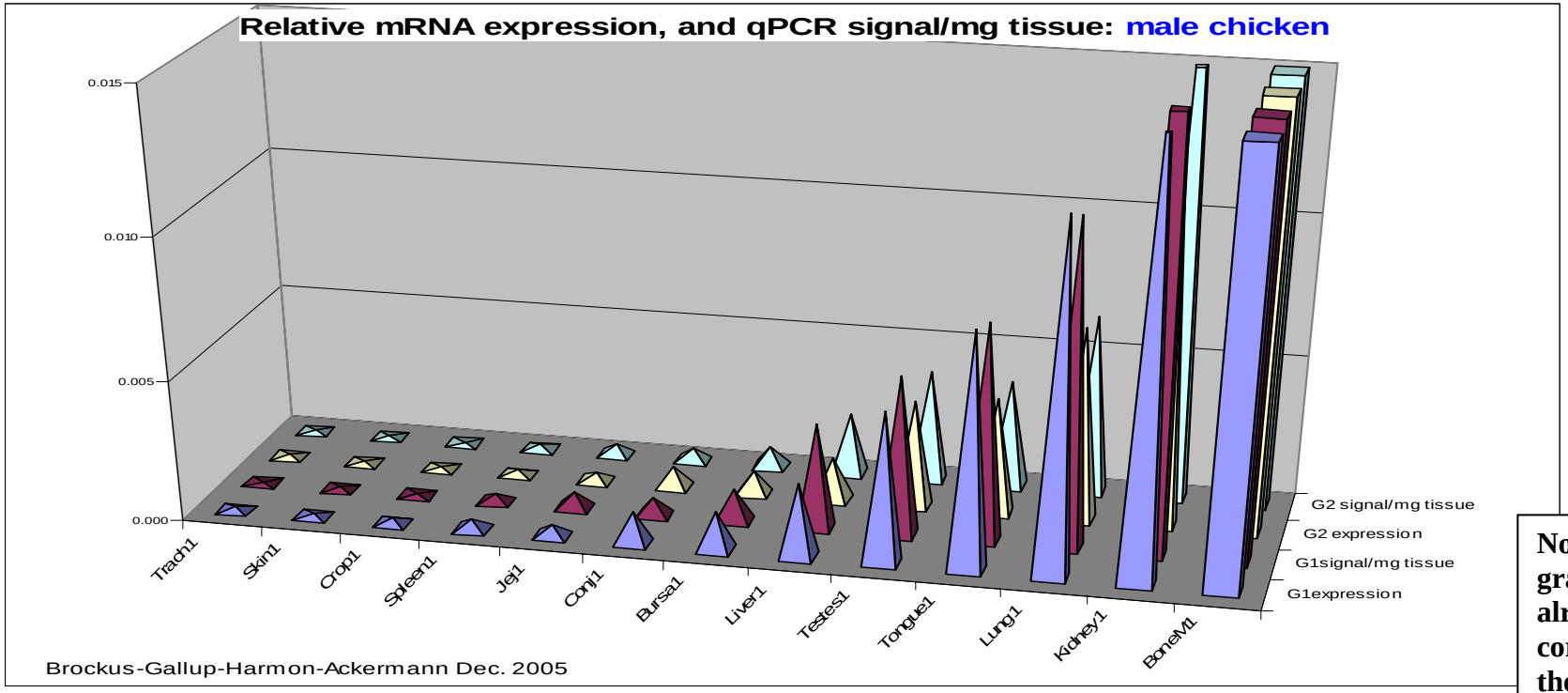
The no RT (NRC) control plate showed 18S signals (Cts) all >14 cycles or more beyond any of the sample 18S signals in all cases. So there are no superfluous signals of any significance impacting our mRNA signal values for any sample. Any genomic DNA real-time signals for the targets and housekeeper in this study were thus mathematically inconsequential

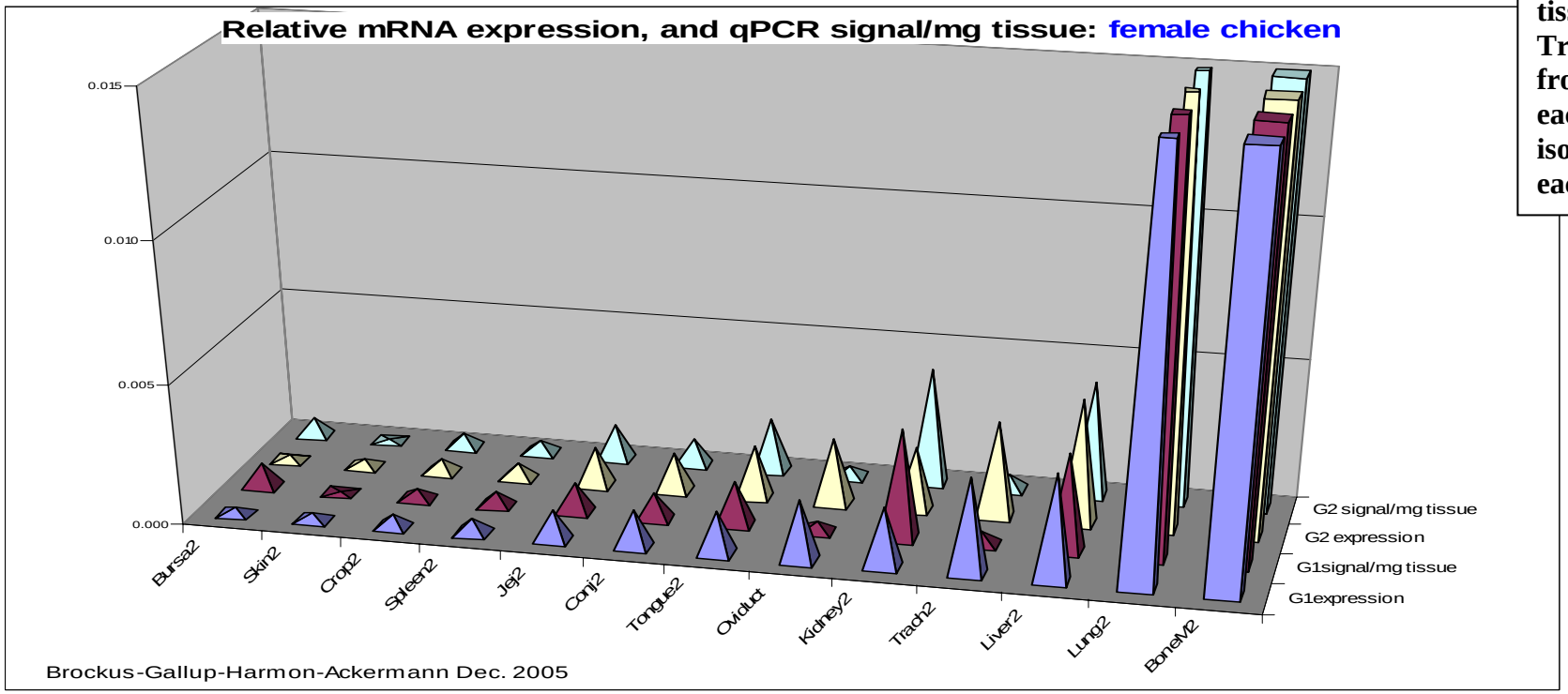


This shows our genomic DNA 18S rRNA signal for all samples … and we have shown this clearly to present no mathematical consequence whatsoever.

~FINAL GRAPHS~

(@ 15 CYCLE BASELINE CUTOFF)

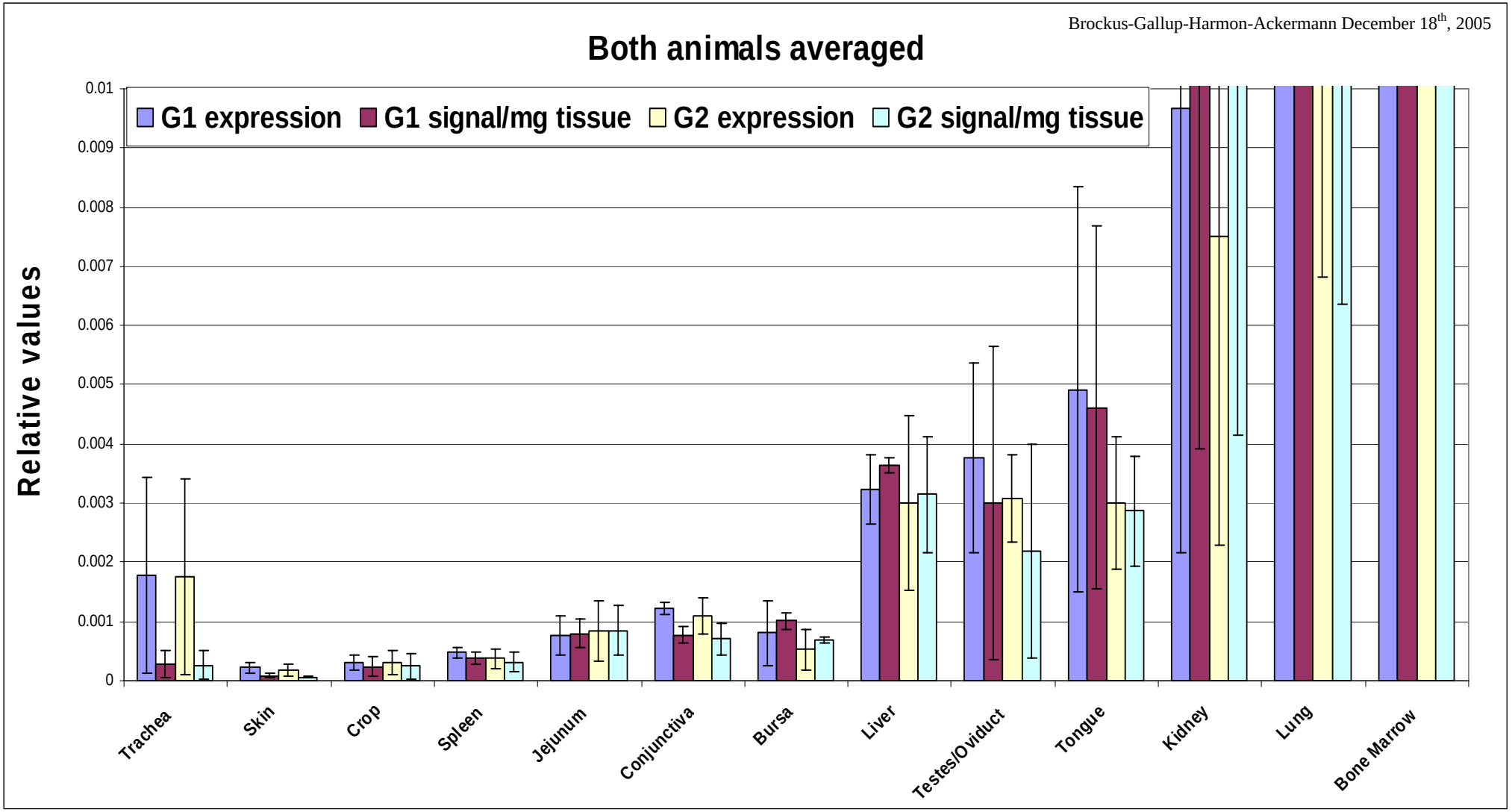




Note: These graphs have already been corrected to the exact mg tissue/mL Trizol slurry from which each RNA was isolated in each case

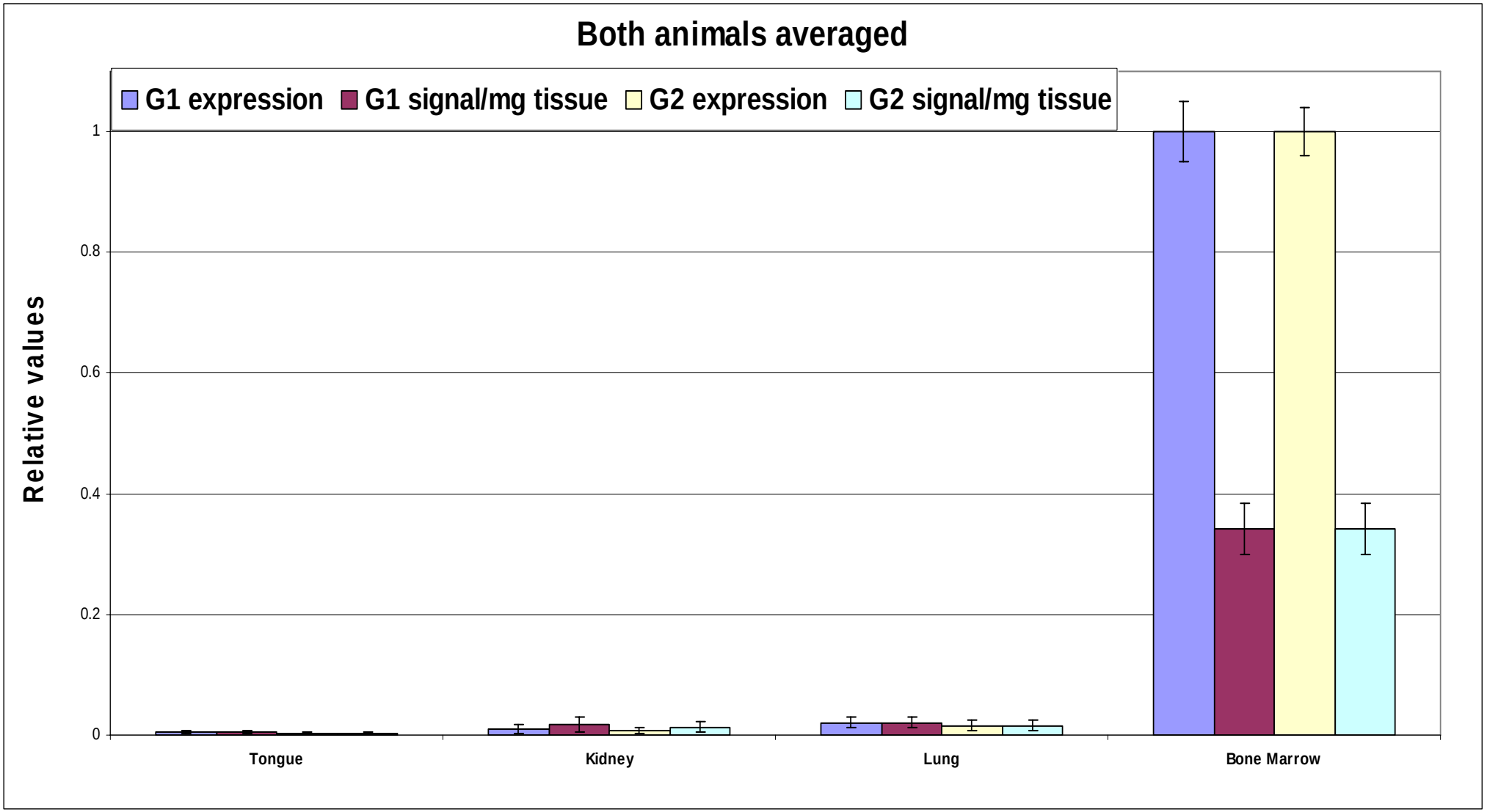
Graphs showing relative qPCR signal for G1 and G2 in each tissue, and relative amount of qPCR signal generated per mg of tissue

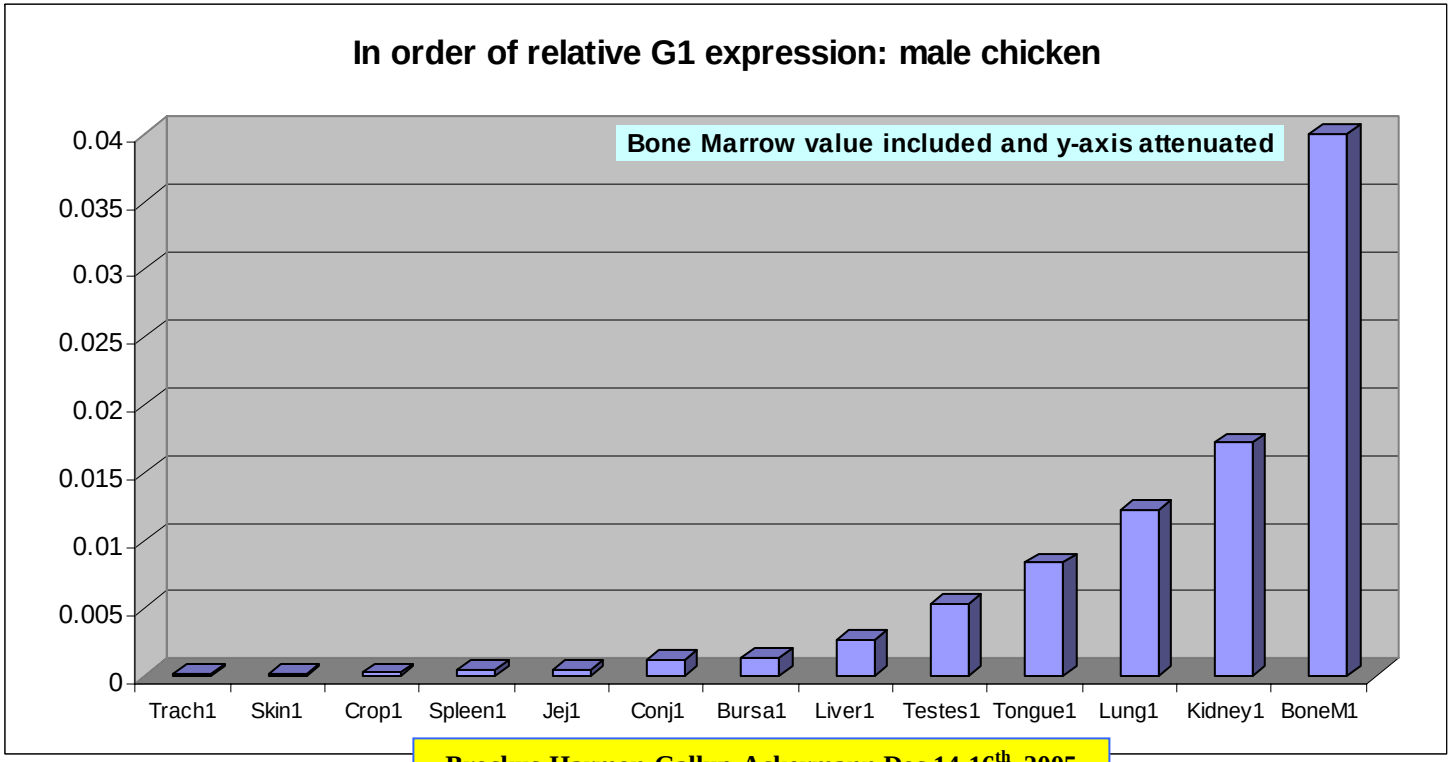
Graphs showing the standard error of the mean (s.e.m.) computed when both chickens' tissue values are averaged:



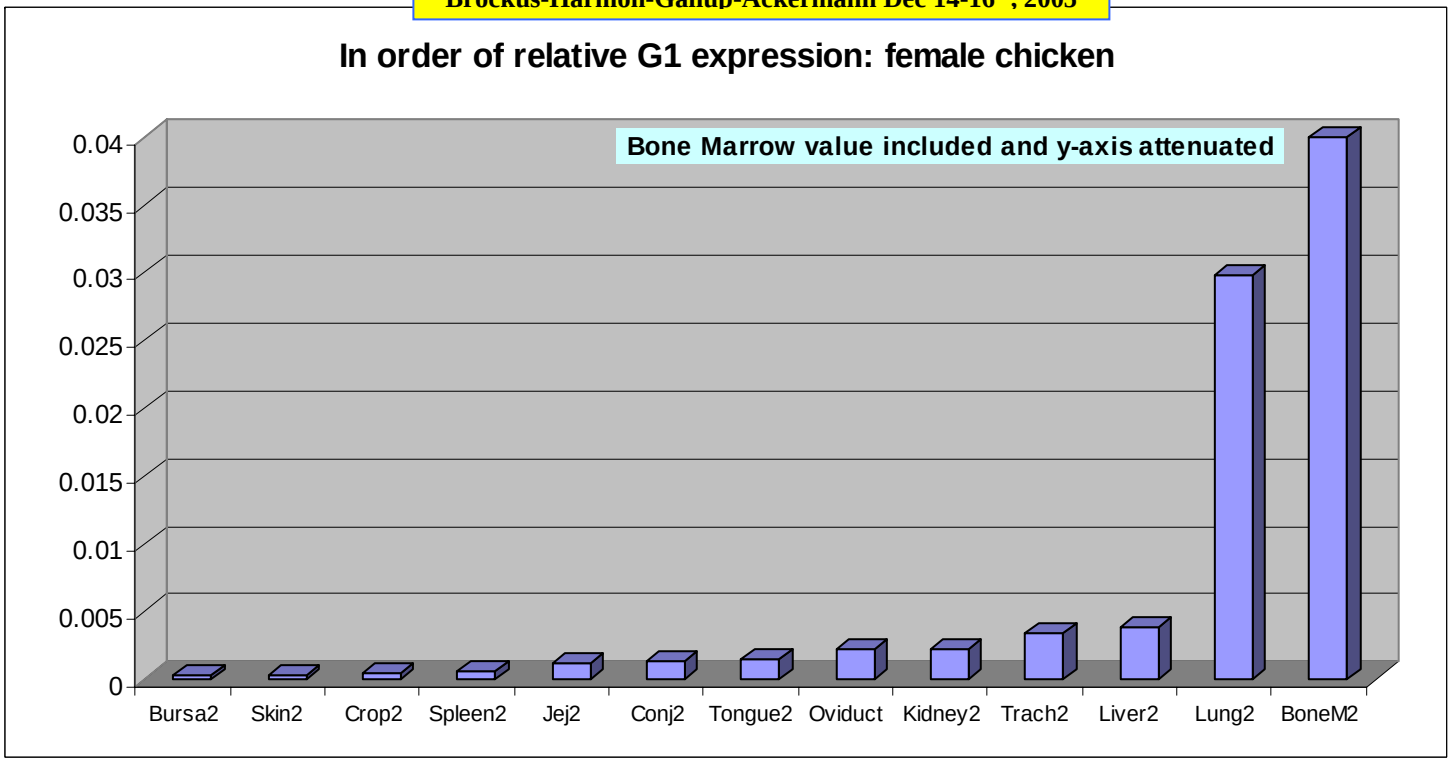
We may have additionally gotten lucky in that Gallus gallus ribosomal 18S RNA seems to be a very stable housekeeper – given what Chuck's Northern work has shown in the past (i.e. the trend of tissue abundance of G1 and G2 seeming to agree very well with logic and prior Northern analyses). Averaging the two chickens in this study may not be necessary – nonetheless, some interesting things are revealed this way. Hopefully Chuck will find some inflammation in the female chicken lungs histologically; this would help to explain the higher tracheal expression of G1 and G2 in the female trachea … etc. …

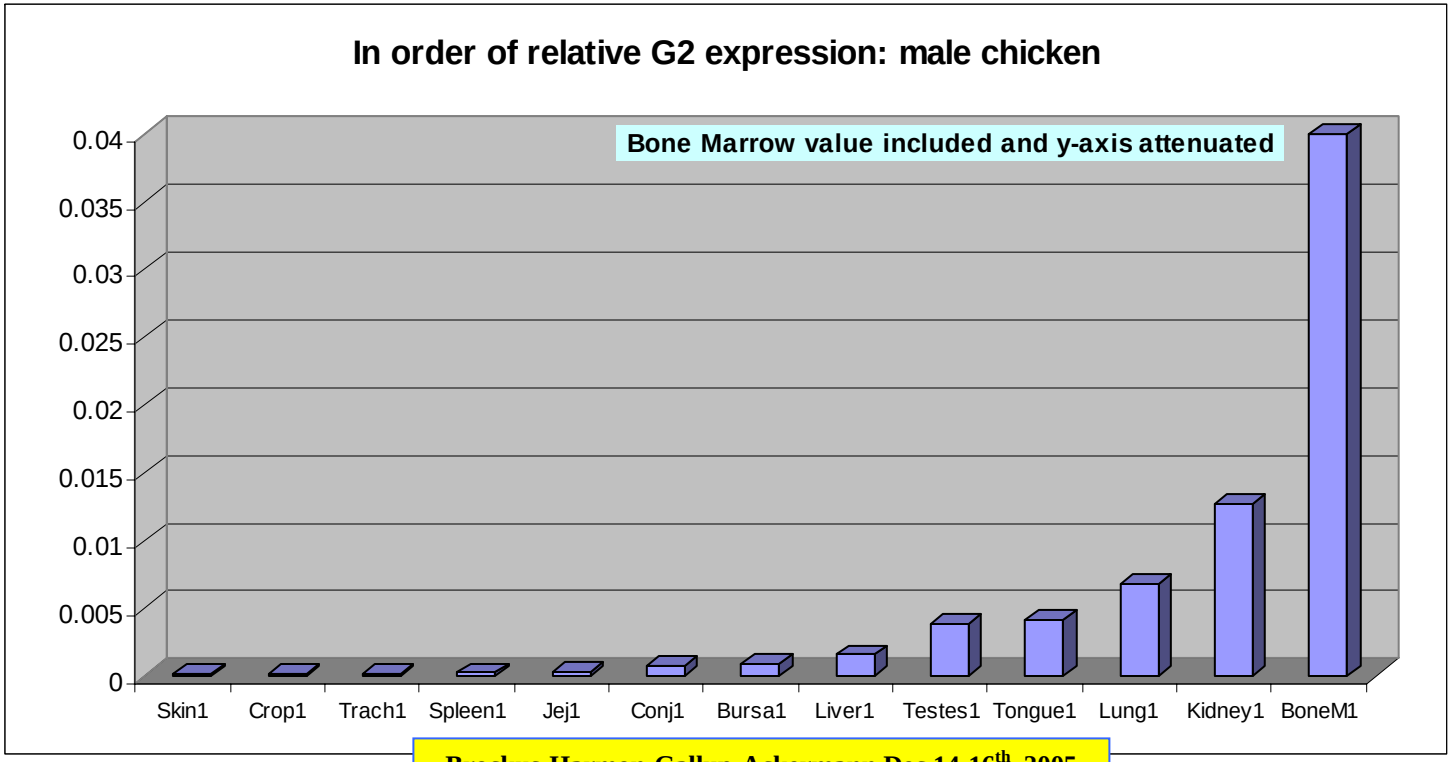
Previous graphs showing tongue, kidney, lung and bone marrow samples more fully:



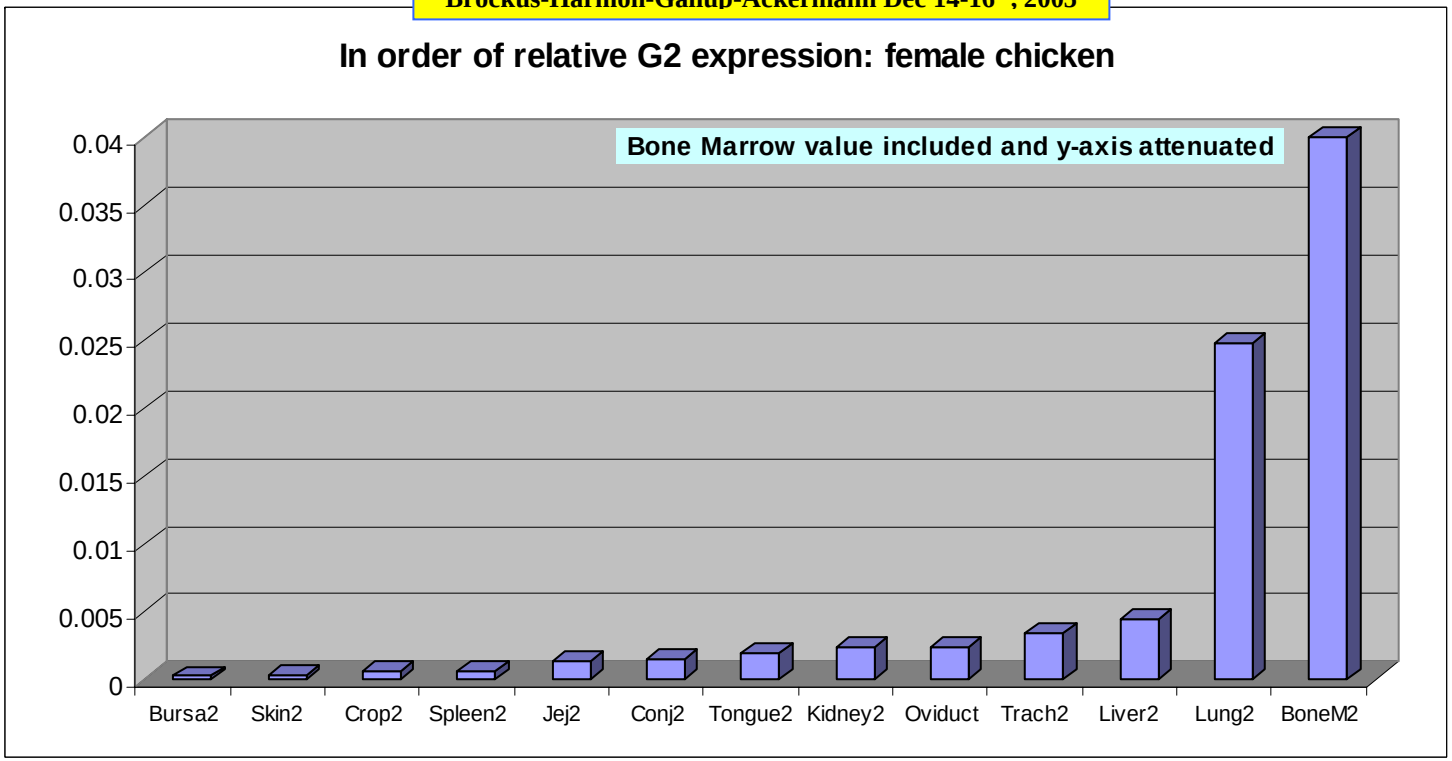


Brockus-Harmon-Gallup-Ackermann Dec 14-16th, 2005



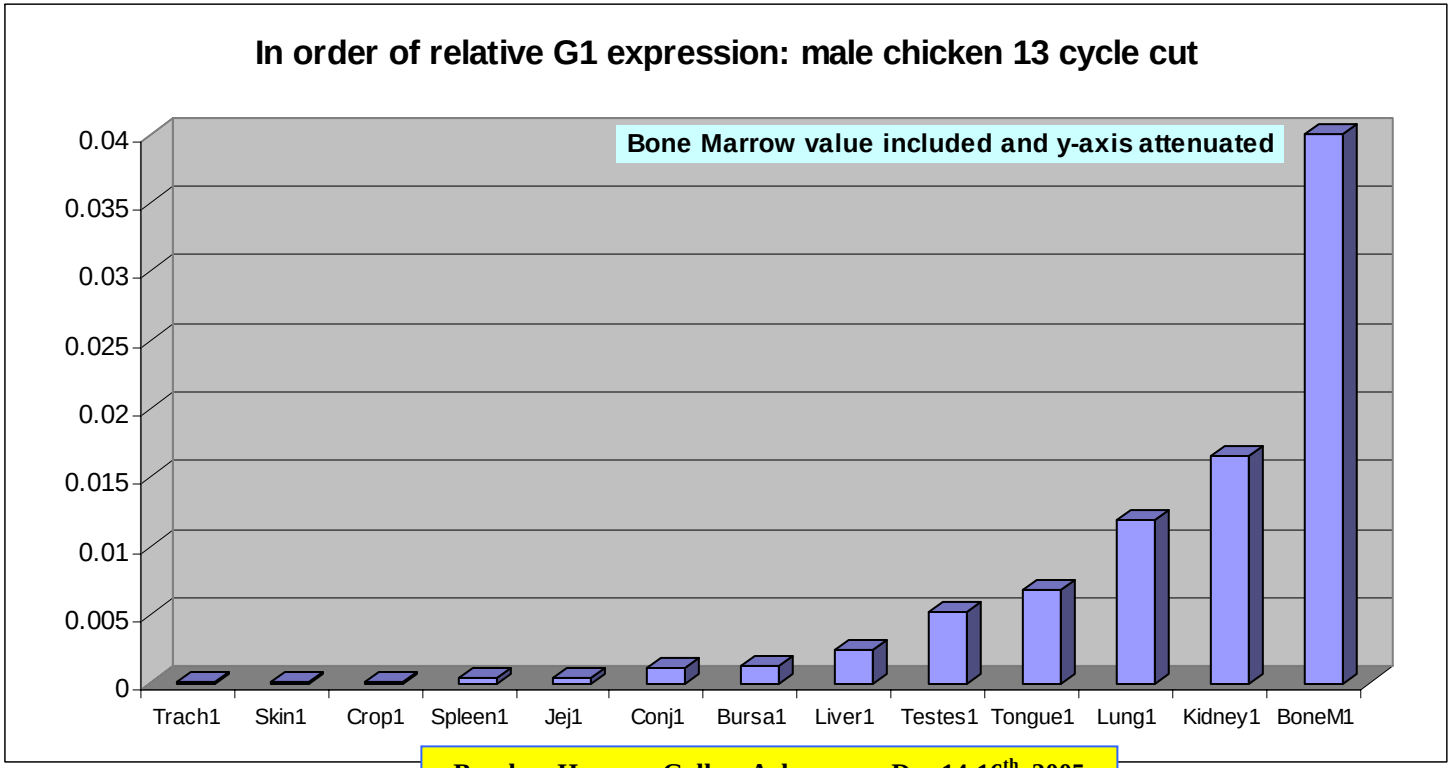


Brockus-Harmon-Gallup-Ackermann Dec 14-16th, 2005

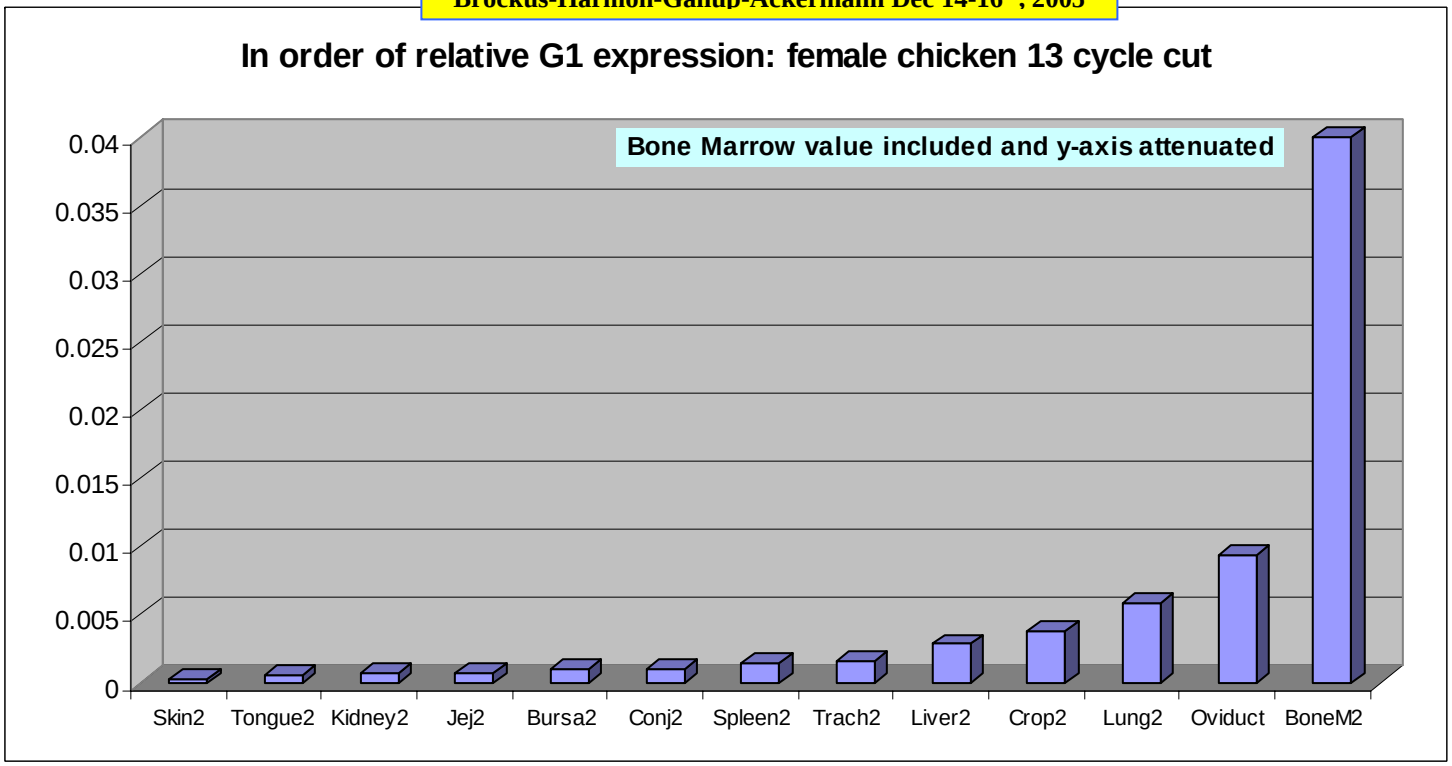


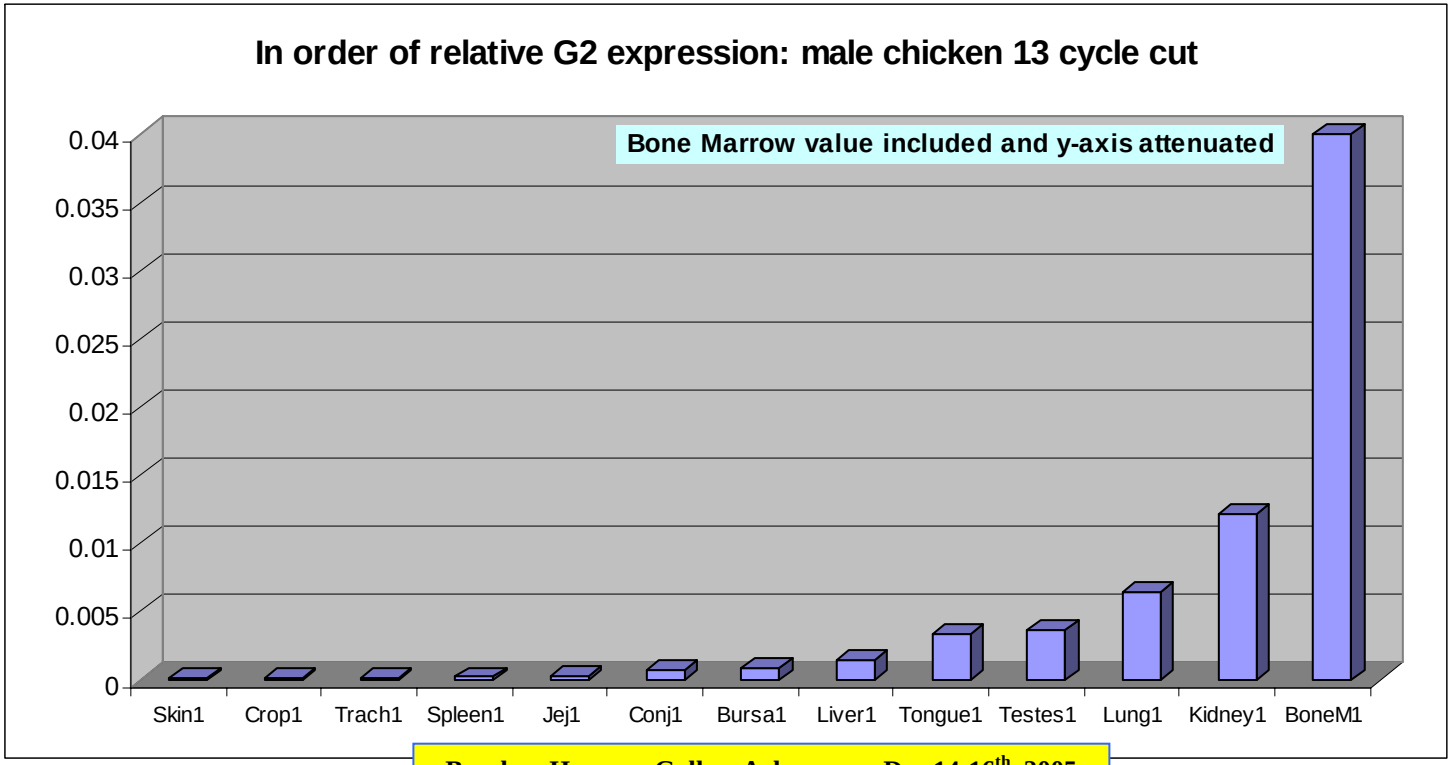
~GRAPHS & RESULTS~

(@ 13 CYCLE BASELINE CUTOFF)

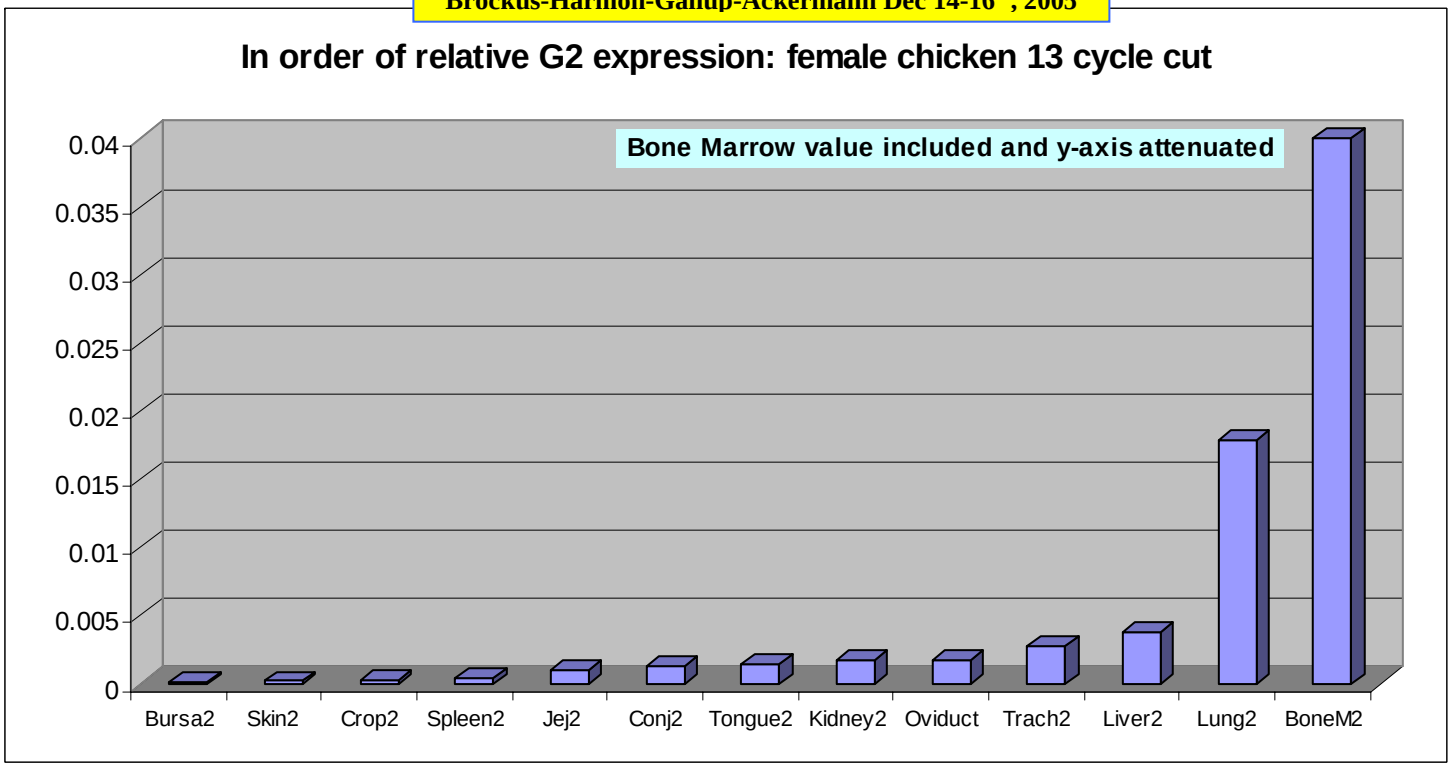


Brockus-Harmon-Gallup-Ackermann Dec 14-16th, 2005

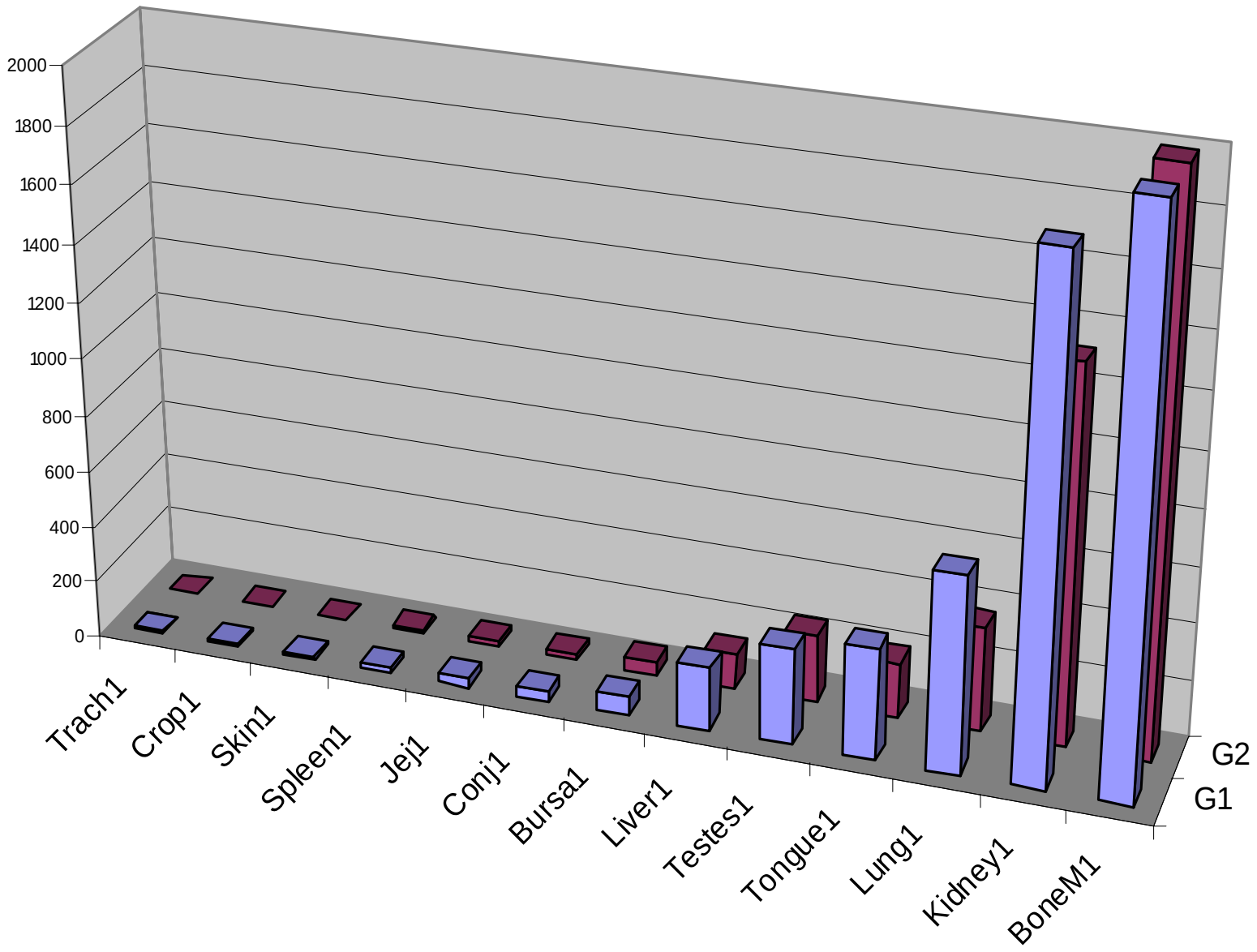




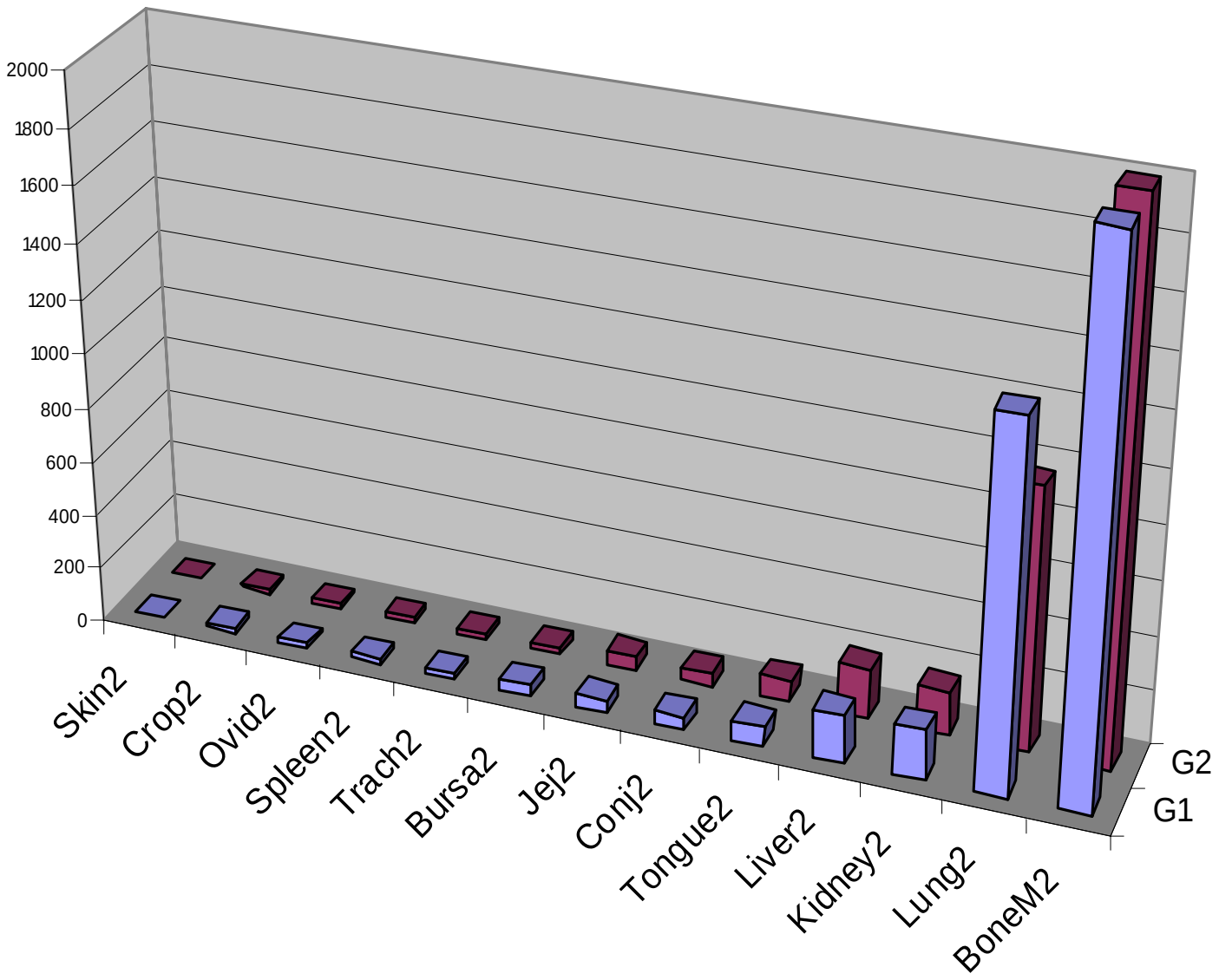
Brockus-Harmon-Gallup-Ackermann Dec 14-16th, 2005

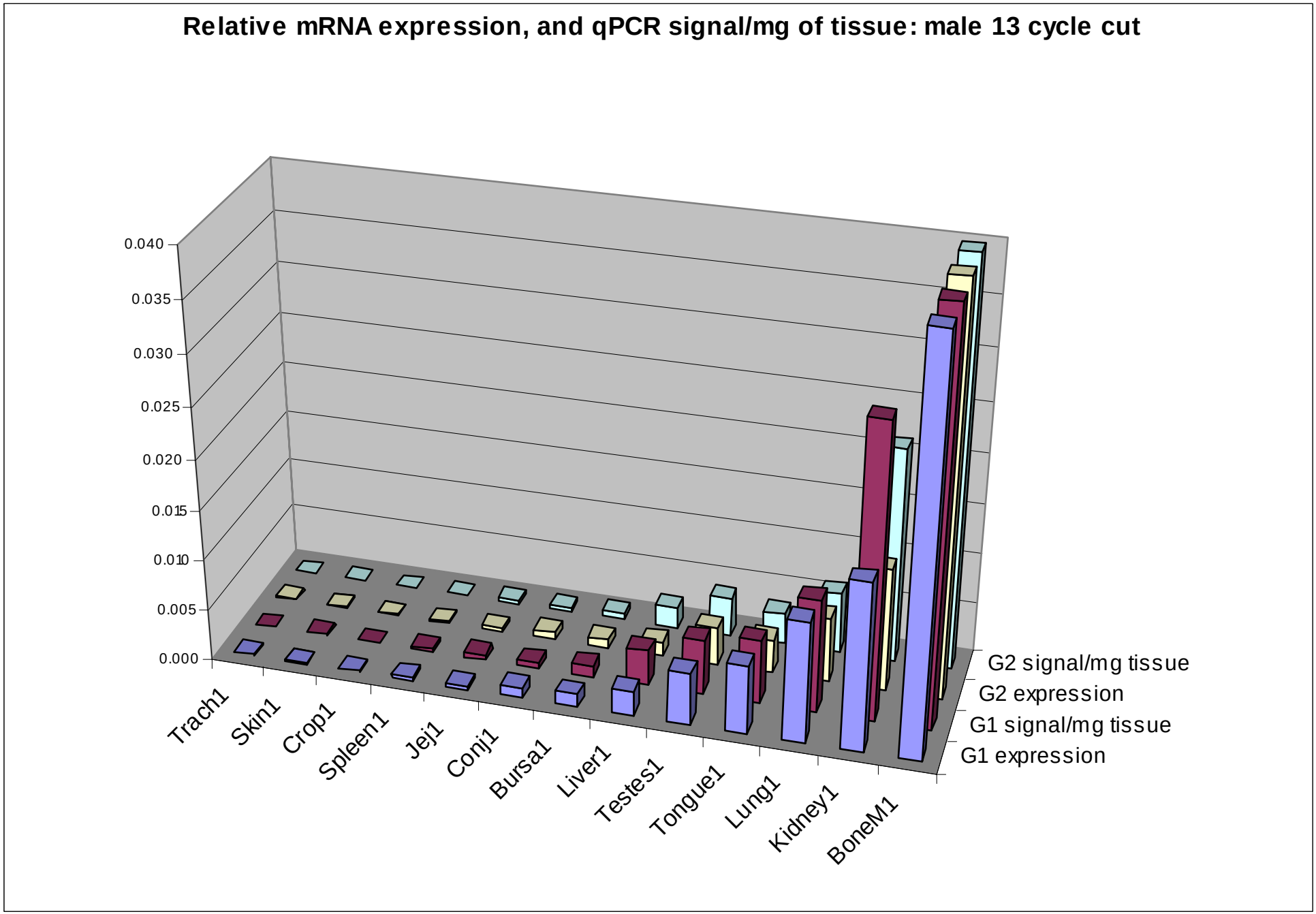


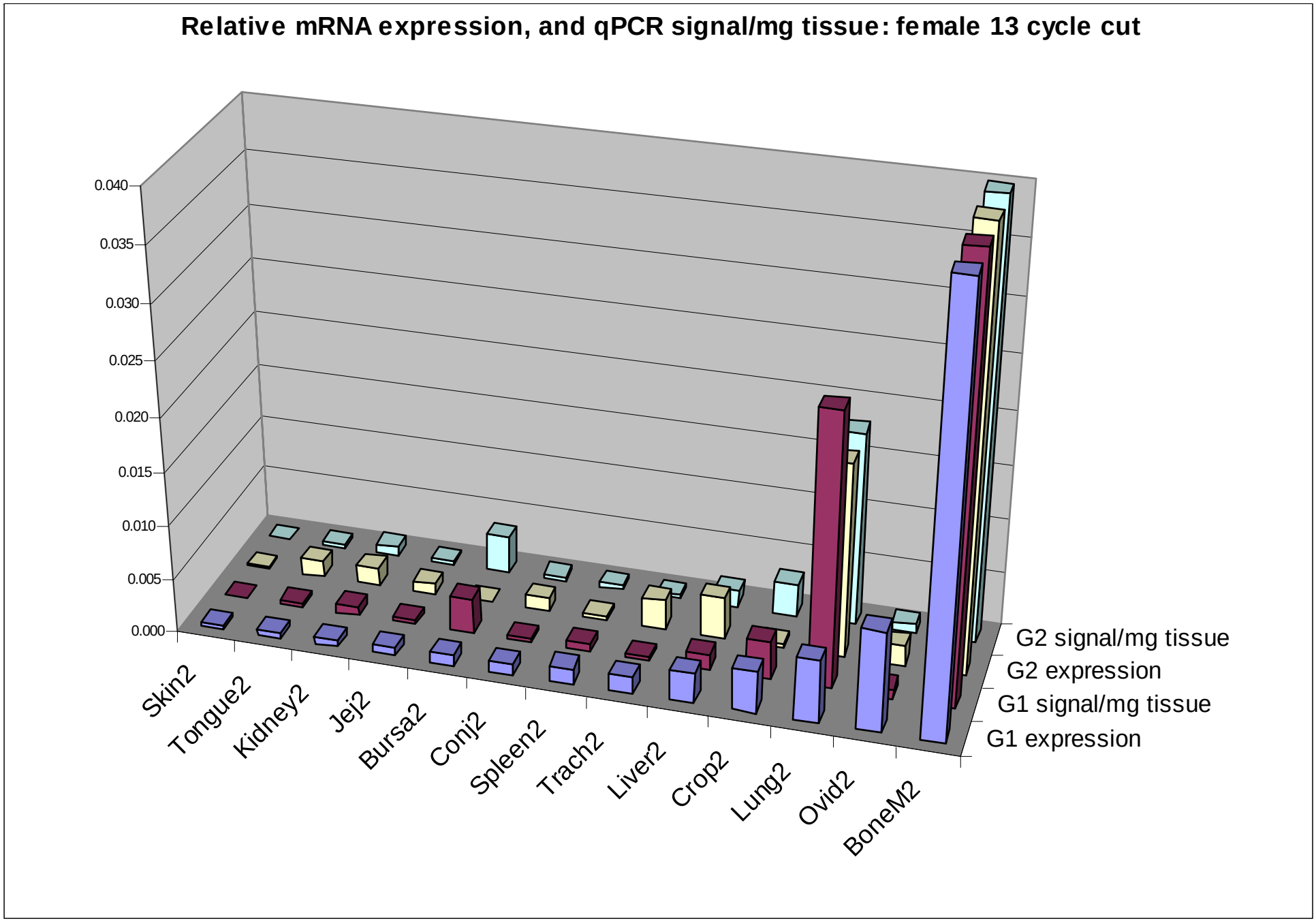
Real-Time target signal per mg of tissue: male chicken 13 cycle cut

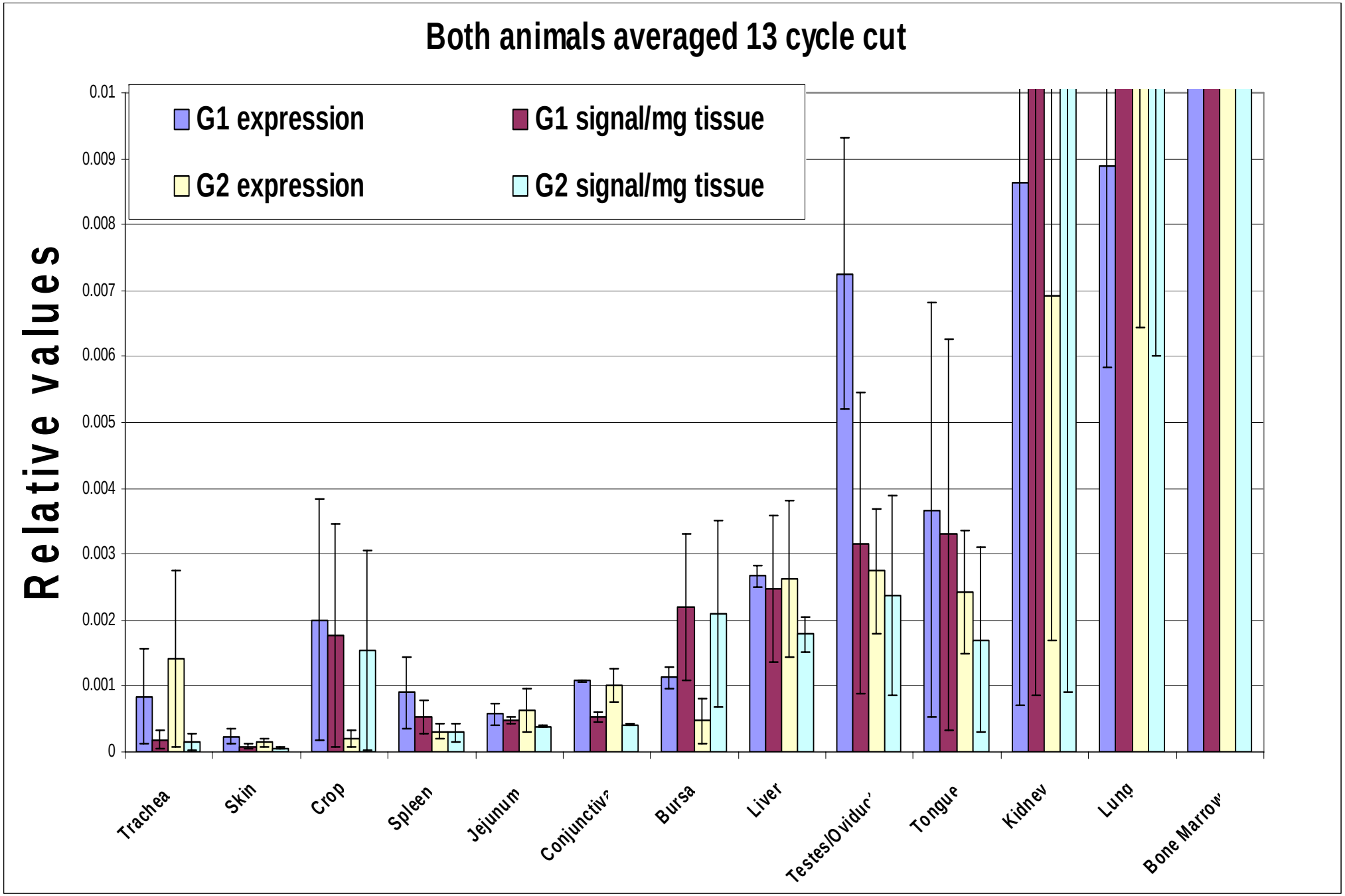


Real-Time target signal per mg of tissue: female chicken 13 cycle cut

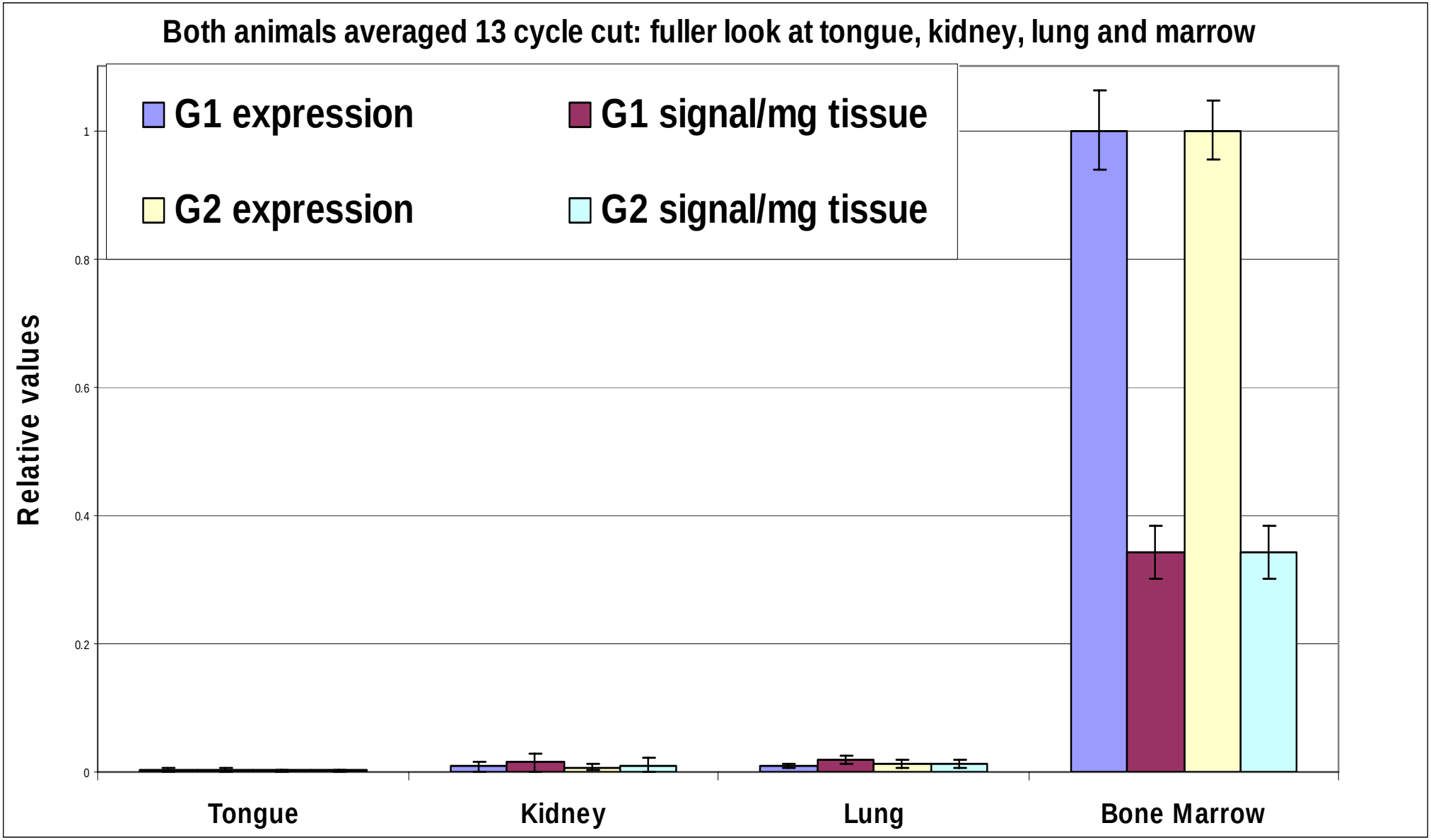


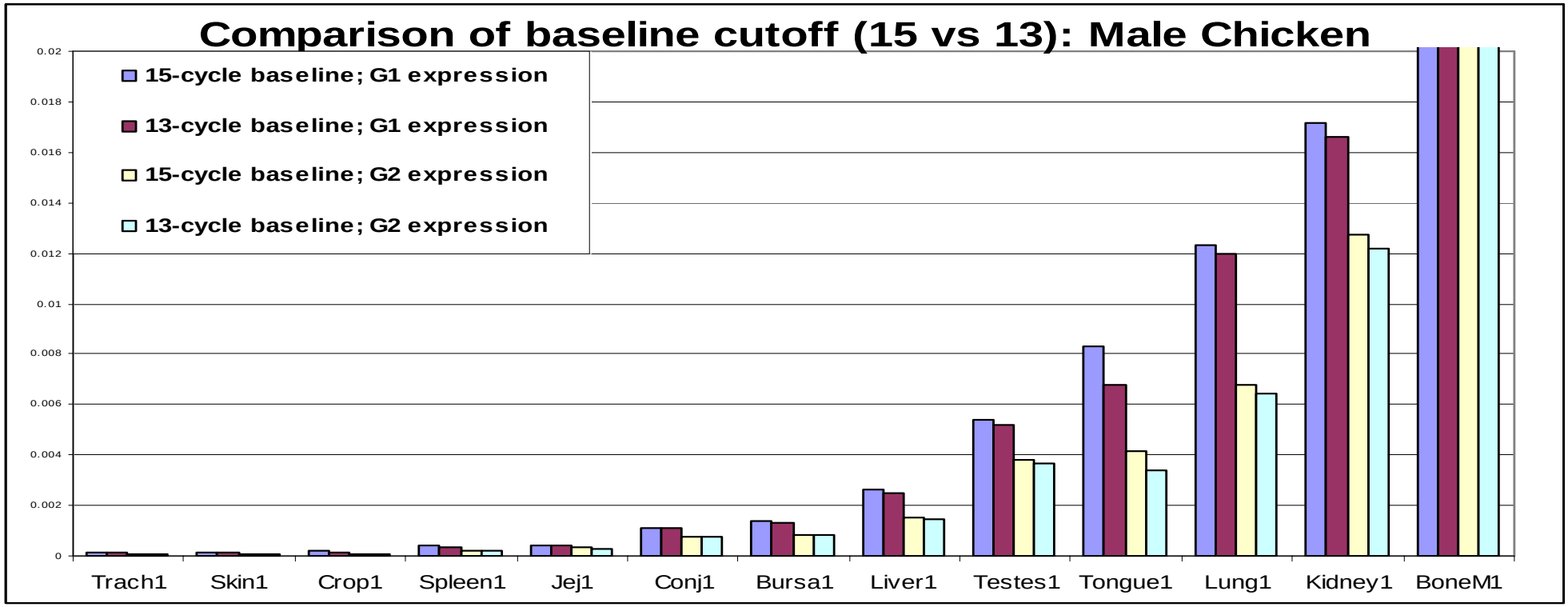

Relative mRNA expression, and qPCR signal/mg of tissue: male 13 cycle cut
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Trach1
Skin1
Crop1
Spleen1
Jej1
Conj1
Bursa1
Liver1
Testes1
Tongue1
Lung1
Kidney1
BoneM1
G2 signal/mg tissue
G2 expression
G1 signal/mg tissue
G1 expression

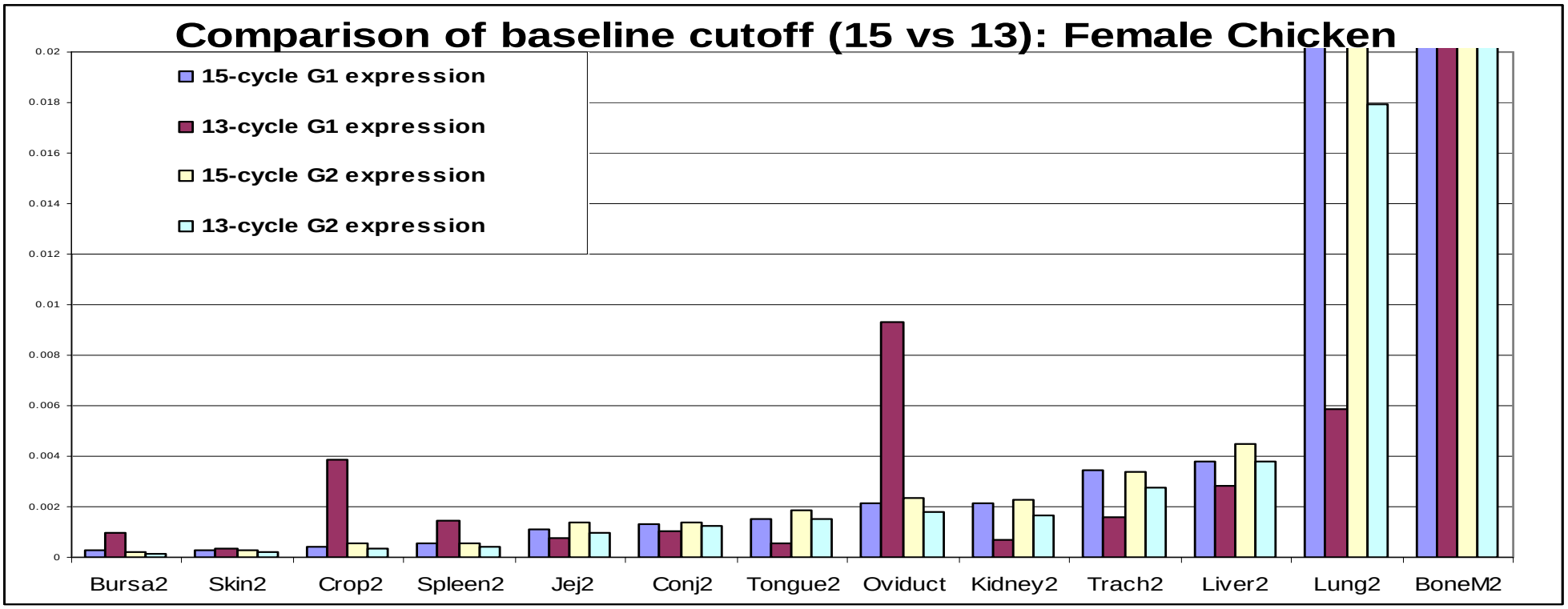

Relative mRNA expression, and qPCR signal/mg tissue: female 13 cycle cut
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Skin2
Tongue2
Kidney2
Jej2
Bursa2
Conj2
Spleen2
Trach2
Liver2
Crop2
Lung2
Ovid2
BoneM2
G2 signal/mg tissue
G2 expression
G1 signal/mg tissue
G1 expression


Both animals averaged 13 cycle cut
G1 expression
G1 signal/mg tissue
G2 expression
G2 signal/mg tissue
Relative values
0.01
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Trachea
Skin
Crop
Spleen
Jejunum
Conjunctiva
Bursa
Liver
Testes/Oviduct
Tongue
Kidney
Lung
Bone Marrow

Previous graphs showing tongue, kidney, lung and bone marrow samples more fully:

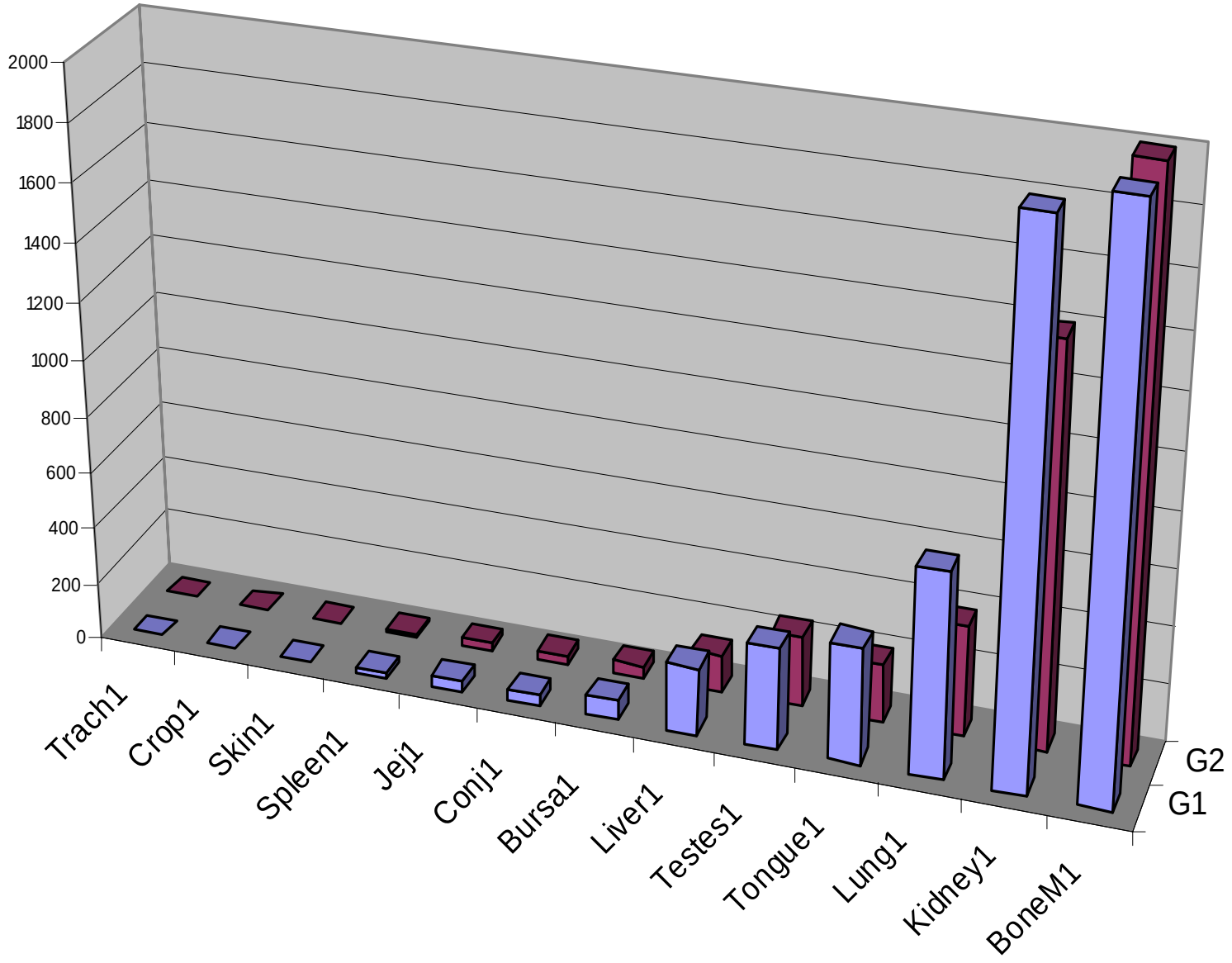



Comparison of baseline cutoff (15 vs 13): Male Chicken
15-cycle baseline; G1 expression
13-cycle baseline; G1 expression
15-cycle baseline; G2 expression
13-cycle baseline; G2 expression
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Trach1
Skin1
Crop1
Spleen1
Jej1
Conj1
Bursa1
Liver1
Testes1
Tongue1
Lung1
Kidney1
BoneM1

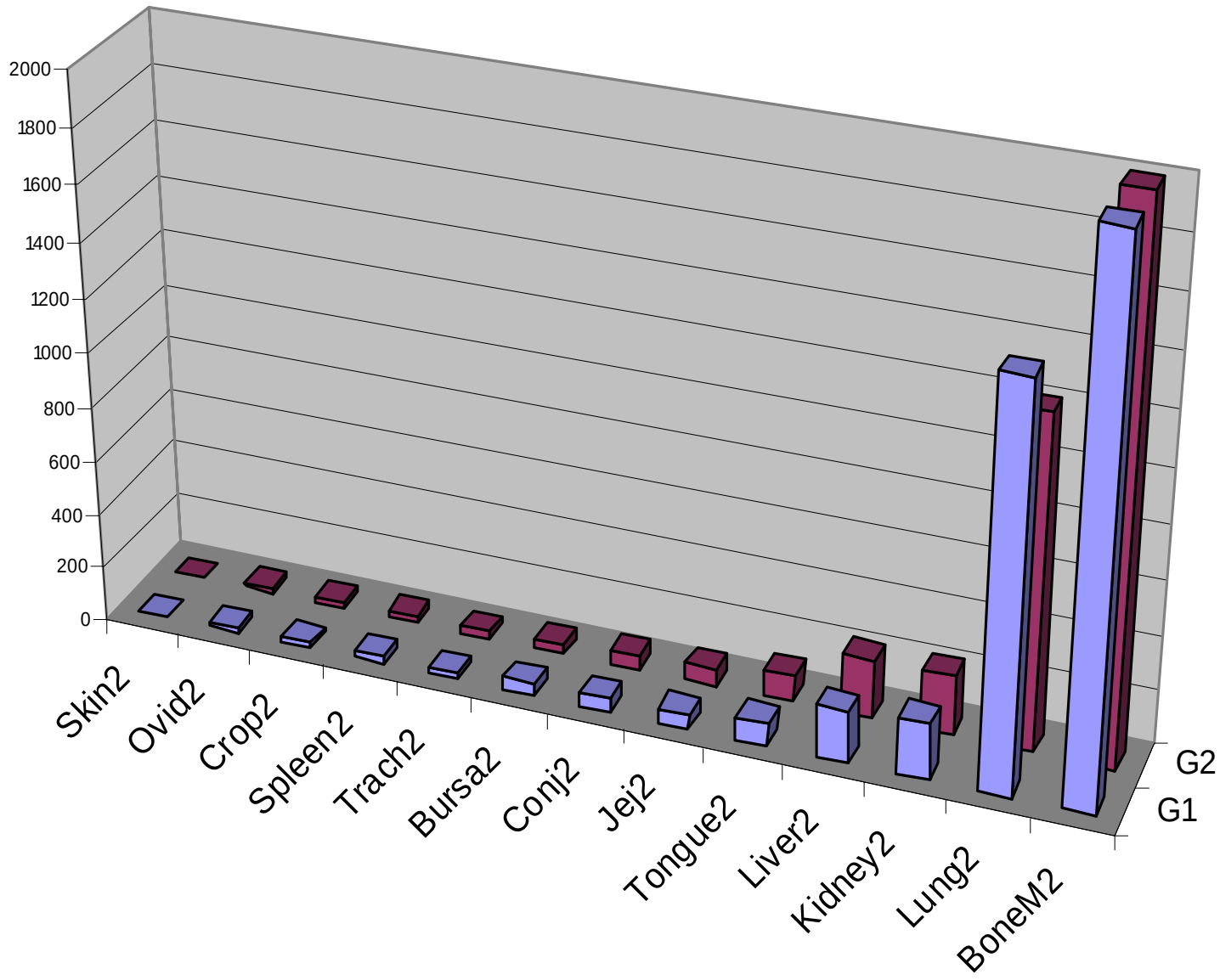

Comparison of baseline cutoff (15 vs 13): Female Chicken
15-cycle G1 expression
13-cycle G1 expression
15-cycle G2 expression
13-cycle G2 expression
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Bursa2
Skin2
Crop2
Spleen2
Jej2
Conj2
Tongue2
Oviduct
Kidney2
Trach2
Liver2
Lung2
BoneM2

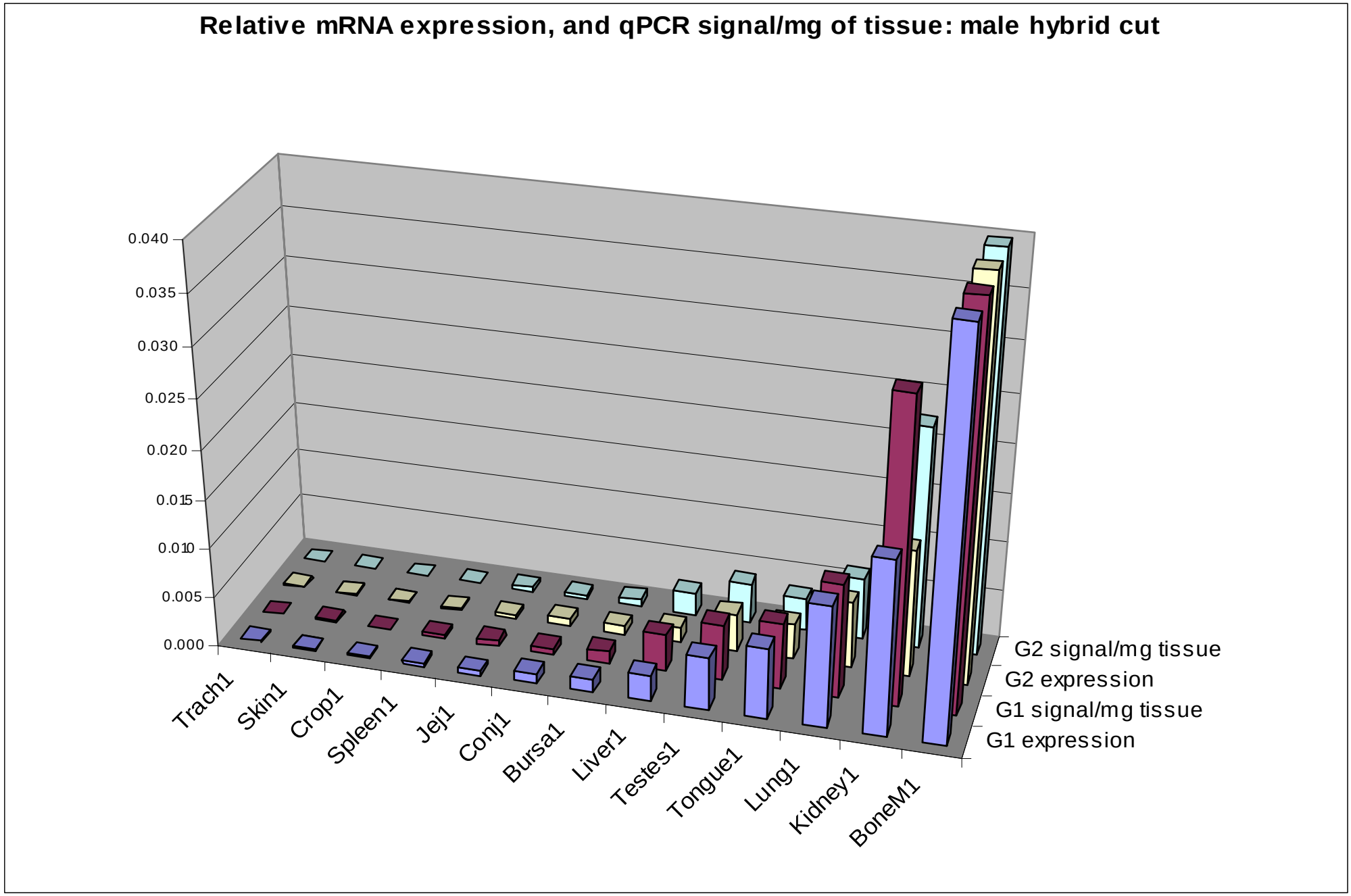
~Hybrid results: 15-cycle baseline cut used for Targets G1 and G2 and a 13-cycle baseline cut used for Housekeeper G18S~

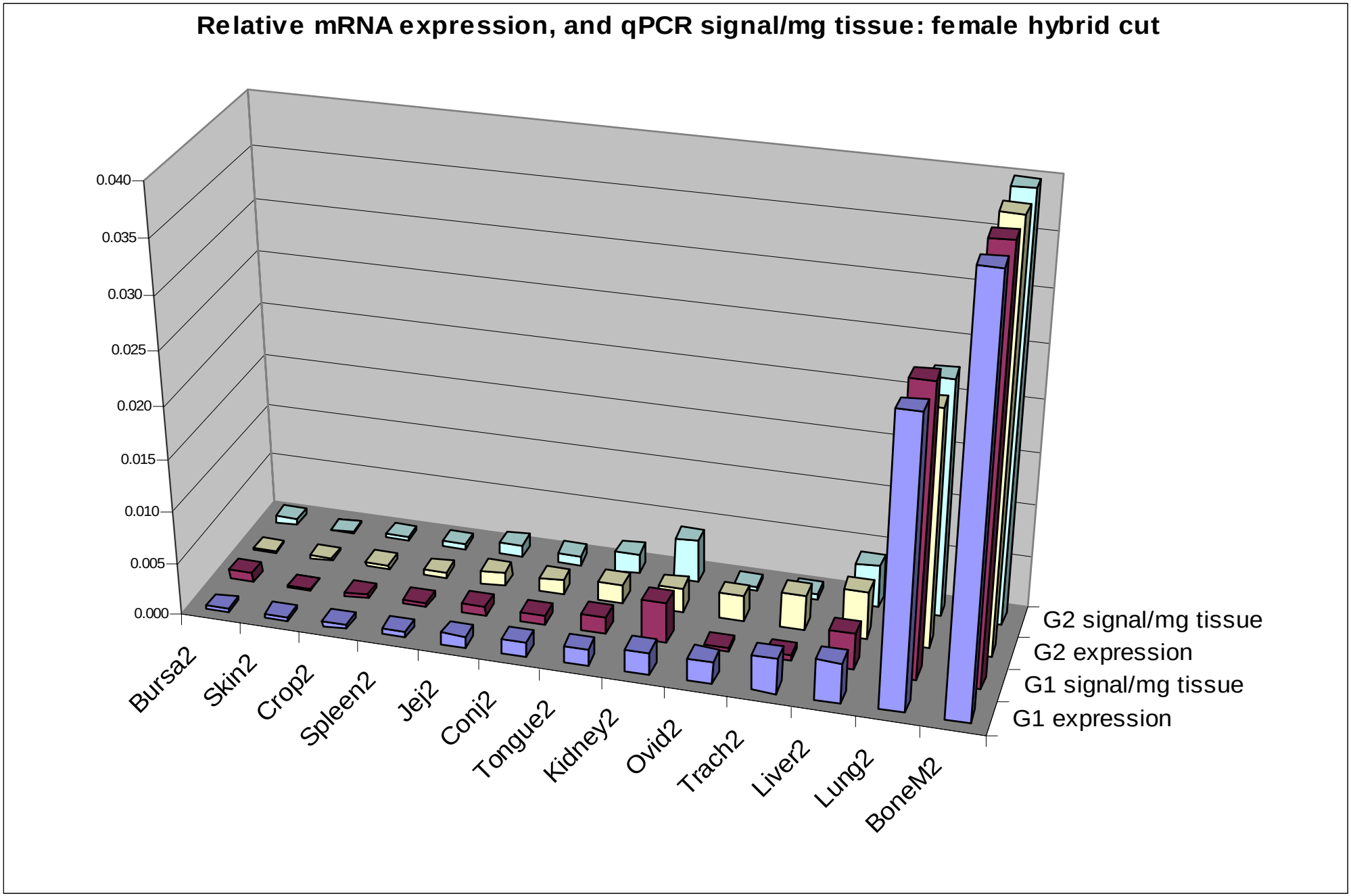
Real-Time target signal per mg of tissue: male chicken hybrid cut

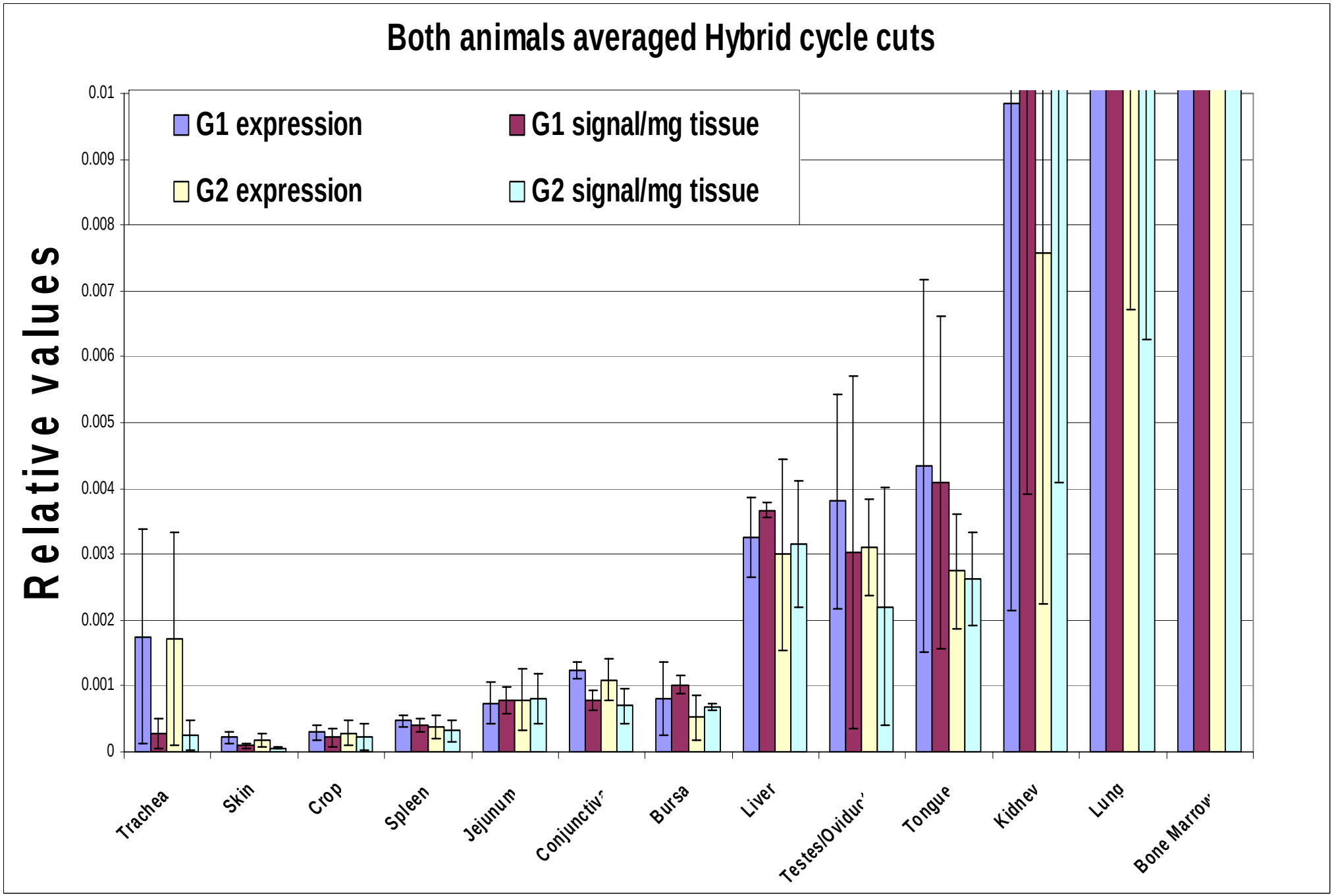


Real-Time target signal per mg of tissue: female chicken hybrid cut




Relative mRNA expression, and qPCR signal/mg of tissue: male hybrid cut
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Trach1
Skin1
Crop1
Spleen1
Jej1
Conj1
Bursa1
Liver1
Testes1
Tongue1
Lung1
Kidney1
BoneM1
G2 signal/mg tissue
G2 expression
G1 signal/mg tissue
G1 expression


Relative mRNA expression, and qPCR signal/mg tissue: female hybrid cut
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
Bursa2
Skin2
Crop2
Spleen2
Jej2
Conj2
Tongue2
Kidney2
Ovid2
Trach2
Liver2
Lung2
BoneM2
G2 signal/mg tissue
G2 expression
G1 signal/mg tissue
G1 expression


Both animals averaged Hybrid cycle cuts
G1 expression
G1 signal/mg tissue
G2 expression
G2 signal/mg tissue
Relative values
0.01
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Trachea
Skin
Crop
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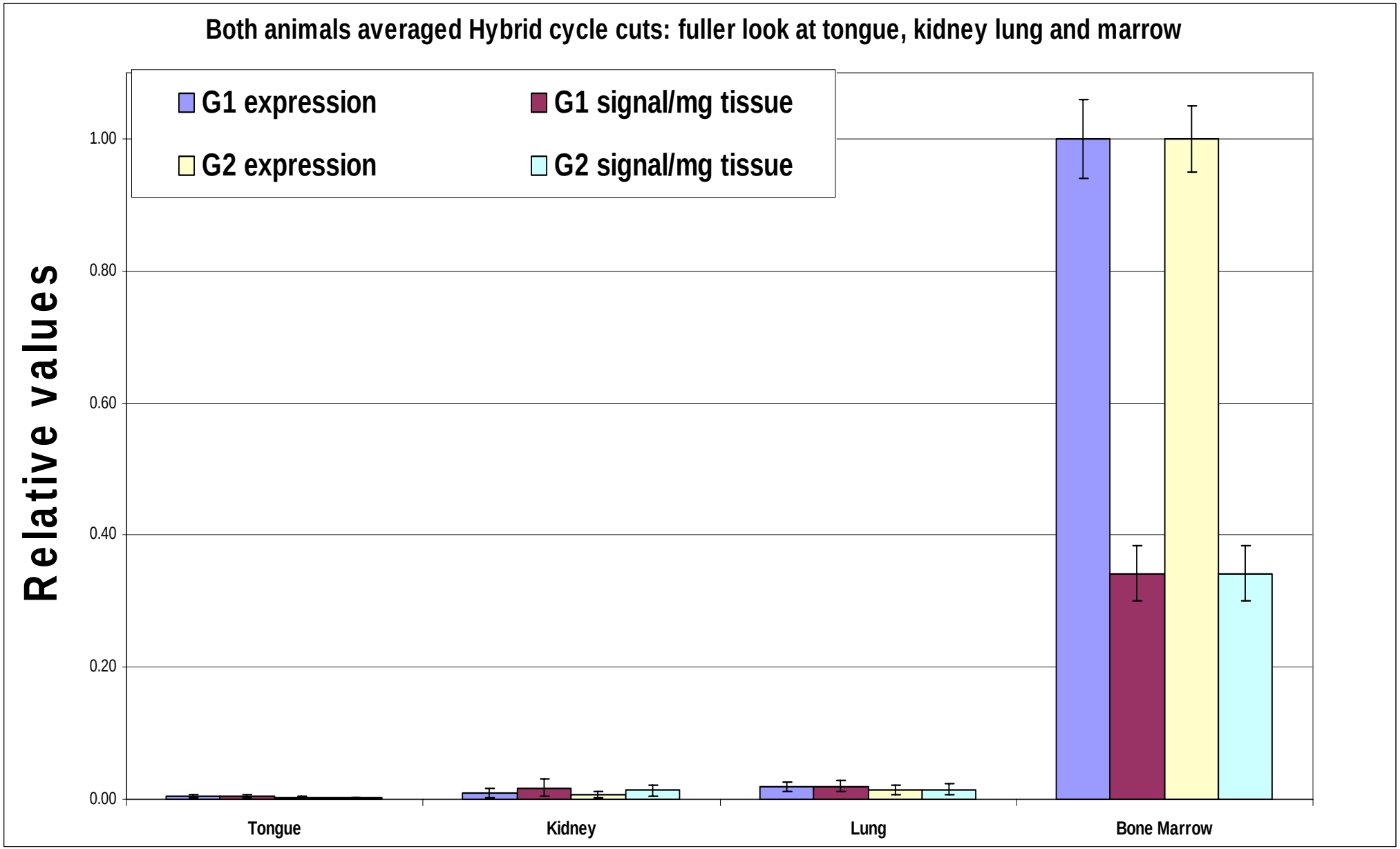
Liver
Testes/Oviduc
Tongue
Kidney
Lung
Bone Marrow

Previous graphs showing tongue, kidney, lung and bone marrow samples more fully:



BUT:

Efficiencies of Reactions

15 cycle baseline cut-off

	Efficiency
Plate 1	
G1:	97.08%
18S:	97.78%
Plate 2	
G1:	103.36%
18S:	98.47%
Plate 3	
G2:	111.32%
18S:	98.55%
Plate 4	
G2:	108.41%
18S:	99.34%

13 cycle baseline cut-off

	Efficiency
Plate 1	
G1:	98.25%
18S:	91.36%
Plate 2	
G1:	106.07%
18S:	91.94%
Plate 3	
G2:	111.94%
18S:	91.53%
Plate 4	
G2:	115.50%
18S:	92.91%

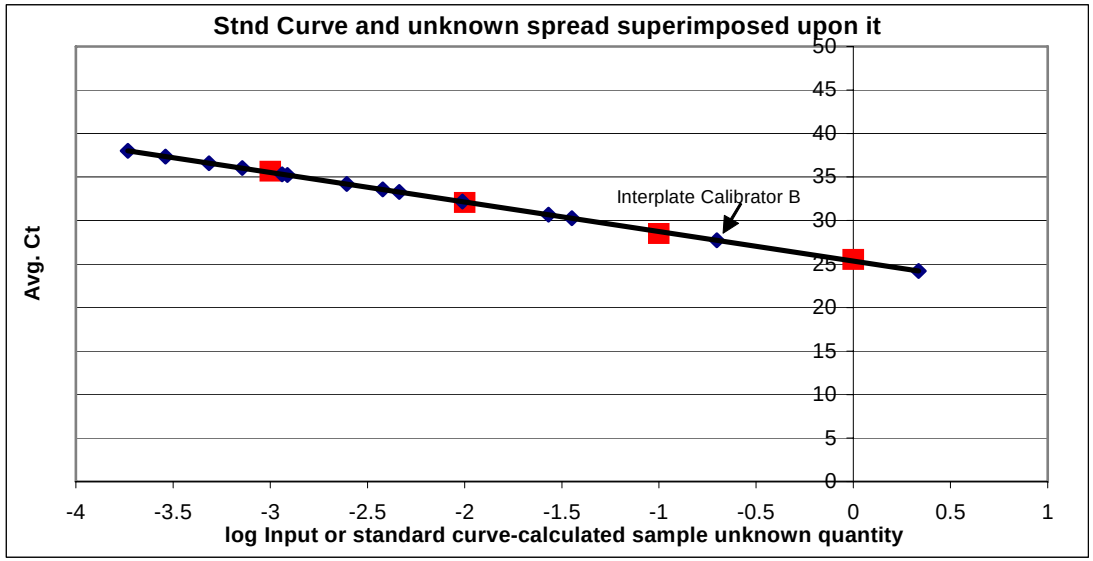
These are better to use: more similar

A choice is made to stick with one data set

~THE UNKNOWN SPREADS SUPERIMPOSED OVER THEIR RESPECTIVE TARGET STANDARD CURVES~

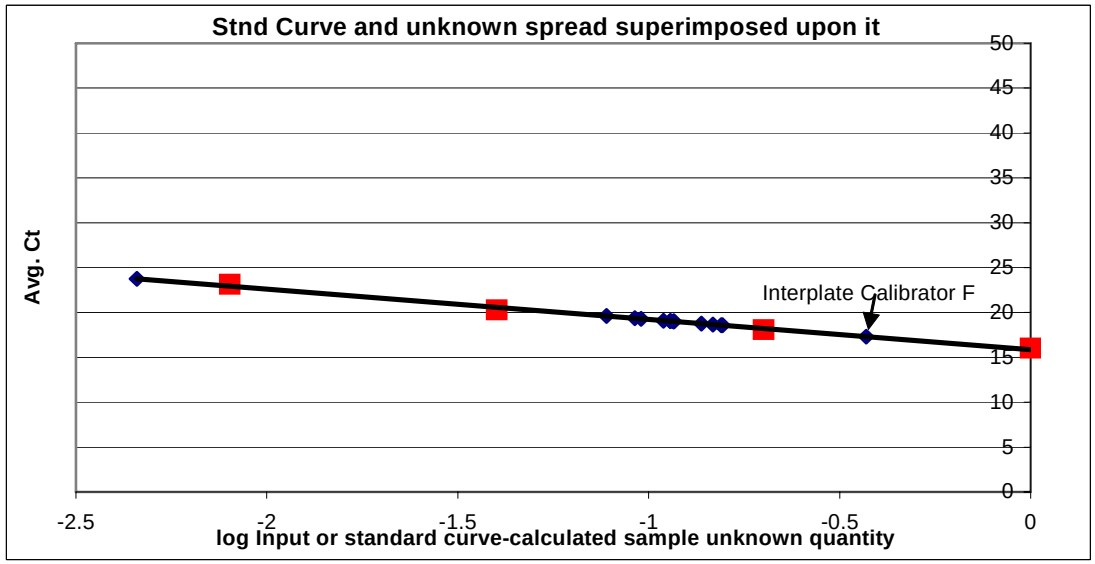
(A direct look at the excellent fidelity established in this study)

	G1 NTC		50.00
		Log of input or Q	Av. Ct
	Stnd1	0	25.53
	Stnd2	-1	28.51
	Stnd3	-2	32.06
	Stnd4	-3	35.66
0.198317	CALB	-0.702641	27.74
2.162199	1	0.334896	24.21
0.001227	2	-2.911052	35.23
0.000485	3	-3.314228	36.60
0.009736	4	-2.011631	32.18
0.027009	5	-1.568493	30.67
0.000289	6	-3.538552	37.38
0.001152	7	-2.938478	35.32
0.004597	8	-2.337507	33.28
0.03569	9	-1.447452	30.26
0.003796	10	-2.420707	33.57
0.000185	11	-3.732652	38.02
0.002477	12	-2.606	34.20
0.000719	13	-3.143234	36.03



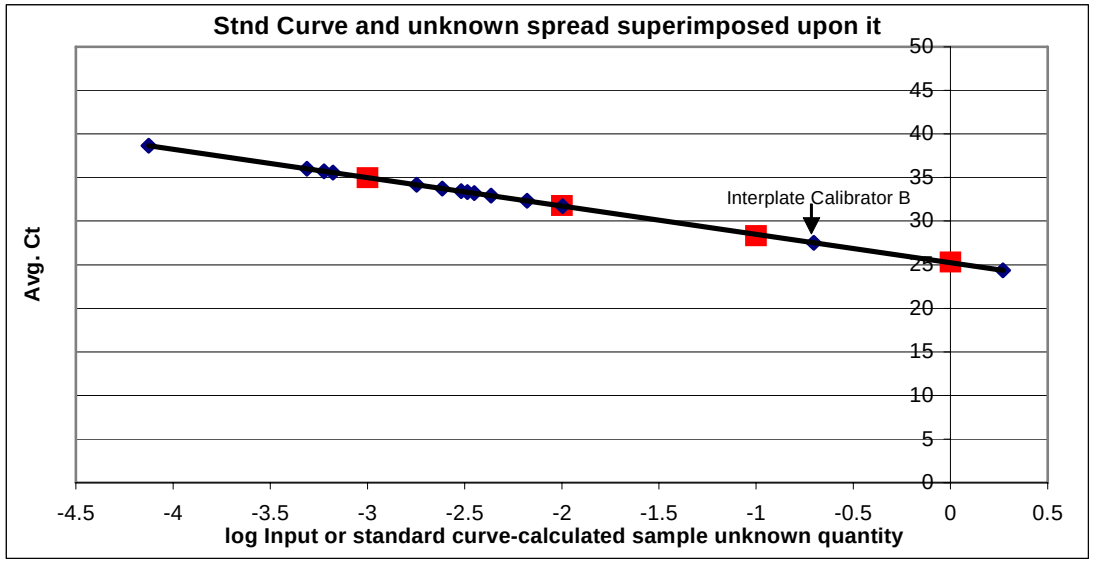
■ = standard curve points
◆ = unknown samples

	18S NTC		36.74
		Log input or Qty	Av. Ct
	Stnd1	0	16.04
	Stnd2	-0.69897	18.09
	Stnd3	-1.39794	20.32
	Stnd4	-2.09691	23.16
0.370844	CALF	-0.430809	17.32
0.113943	1	-0.943311	19.05
0.154859	2	-0.810063	18.60
0.13745	3	-0.861855	18.77
0.095434	4	-1.020296	19.31
0.115969	5	-0.935657	19.02
0.113959	6	-0.94325	19.05
0.15593	7	-0.807071	18.59
0.091935	8	-1.036517	19.36
0.109375	9	-0.961081	19.11
0.147202	10	-0.832087	18.67
0.077525	11	-1.110561	19.61
0.11645	12	-0.93386	19.02
0.004569	13	-2.340141	23.77



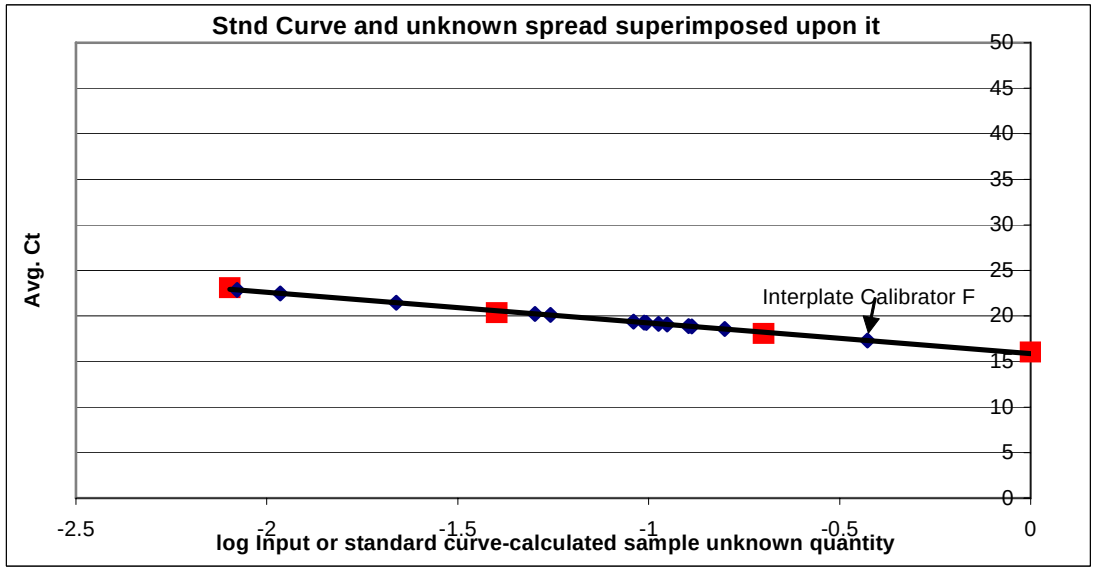
Notice the fine placement of the interplate calibrator – as predicted, and the acceptable spread of unknowns within the bounds of standard-curve linearity for all samples …

	G1 NTC		50.00
		Log of input or Q	Av. Ct
	Stnd1	0	25.33
	Stnd2	-1	28.34
	Stnd3	-2	31.80
	Stnd4	-3	34.99
0.198088	CALB	-0.703142	27.53
1.859791	14	0.269464	24.37
0.000489	15	-3.310705	35.99
7.5E-05	16	-4.124997	38.65
0.004335	17	-2.362966	32.92
0.006612	18	-2.179648	32.32
0.000598	19	-3.223083	35.70
0.001791	20	-2.746849	34.16
0.010095	21	-1.995907	31.72
0.002431	22	-2.614143	33.73
0.000665	23	-3.176984	35.57
0.003542	24	-2.450765	33.20
0.00303	25	-2.518545	33.43
0.003265	26	-2.486163	33.31



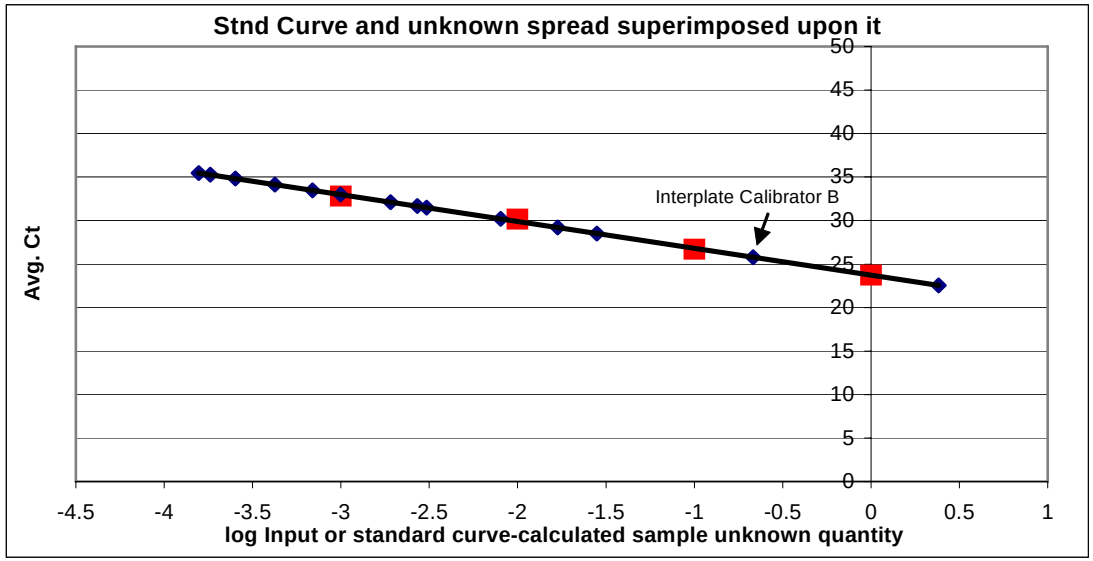
= standard curve points
= unknown samples

	18S NTC		41.93
		Log input or Qty	Av. Ct
	Stnd1	0	16.03
	Stnd2	-0.69897	18.09
	Stnd3	-1.39794	20.36
	Stnd4	-2.09691	23.10
0.374137	CALF	-0.426969	17.31
0.091145	14	-1.040266	19.37
0.021801	15	-1.661515	21.46
0.008362	16	-2.077668	22.85
0.09862	17	-1.006035	19.25
0.01085	18	-1.964578	22.47
0.097286	19	-1.011947	19.27
0.158263	20	-0.80062	18.56
0.129757	21	-0.886868	18.85
0.055264	22	-1.257557	20.10
0.127101	23	-0.895849	18.88
0.050382	24	-1.297727	20.23
0.111636	25	-0.952196	19.07
0.10612	26	-0.974204	19.15



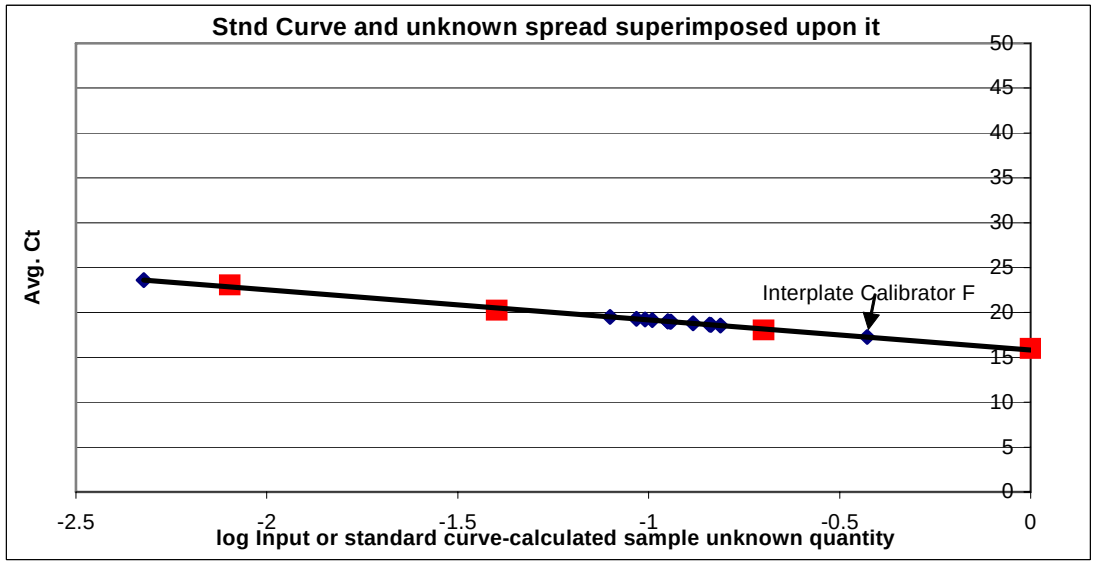
Notice the fine placement of the interplate calibrator – as predicted, and the acceptable spread of unknowns within the bounds of standard-curve linearity for all samples …

	G2 NTC		50.00
		Log of input or Q	Av. Ct
	Stnd1	0	23.72
	Stnd2	-1	26.70
	Stnd3	-2	30.17
	Stnd4	-3	32.82
0.215003	CALB	-0.667555	25.79
2.417995	1	0.383455	22.56
0.000993	2	-3.002847	32.98
0.000253	3	-3.597358	34.85
0.008021	4	-2.095781	30.19
0.016823	5	-1.774091	29.20
0.000183	6	-3.738569	35.25
0.000691	7	-3.160553	33.47
0.003052	8	-2.515439	31.48
0.028101	9	-1.55128	28.51
0.002707	10	-2.567589	31.66
0.000157	11	-3.804888	35.45
0.00191	12	-2.718862	32.11
0.000423	13	-3.373278	34.12



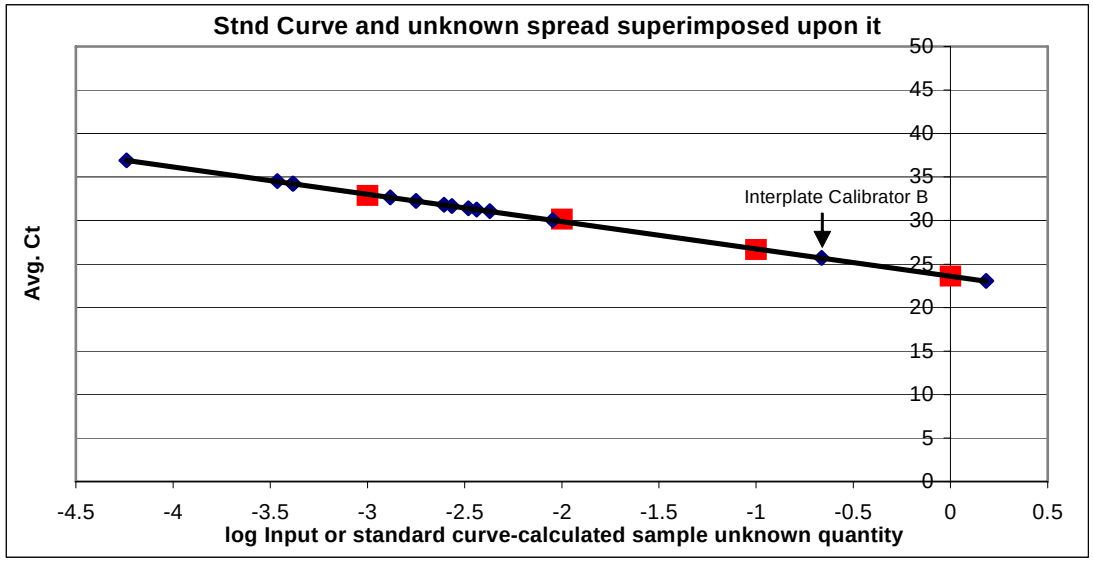
= standard curve points
= unknown samples

	18S NTC		41.20
		Log input or Qty	Av. Ct
	Stnd1	0	15.99
	Stnd2	-0.69897	18.05
	Stnd3	-1.39794	20.27
	Stnd4	-2.09691	23.07
0.373472	CALF	-0.427742	17.26
0.112066	1	-0.950525	19.02
0.144509	2	-0.840106	18.65
0.130794	3	-0.883413	18.79
0.097706	4	-1.01008	19.22
0.114401	5	-0.941568	18.99
0.112072	6	-0.950505	19.02
0.154198	7	-0.811923	18.55
0.09281	8	-1.032403	19.29
0.102157	9	-0.990731	19.15
0.145476	10	-0.837209	18.64
0.07926	11	-1.100946	19.52
0.113668	12	-0.944363	19.00
0.004763	13	-2.322091	23.62



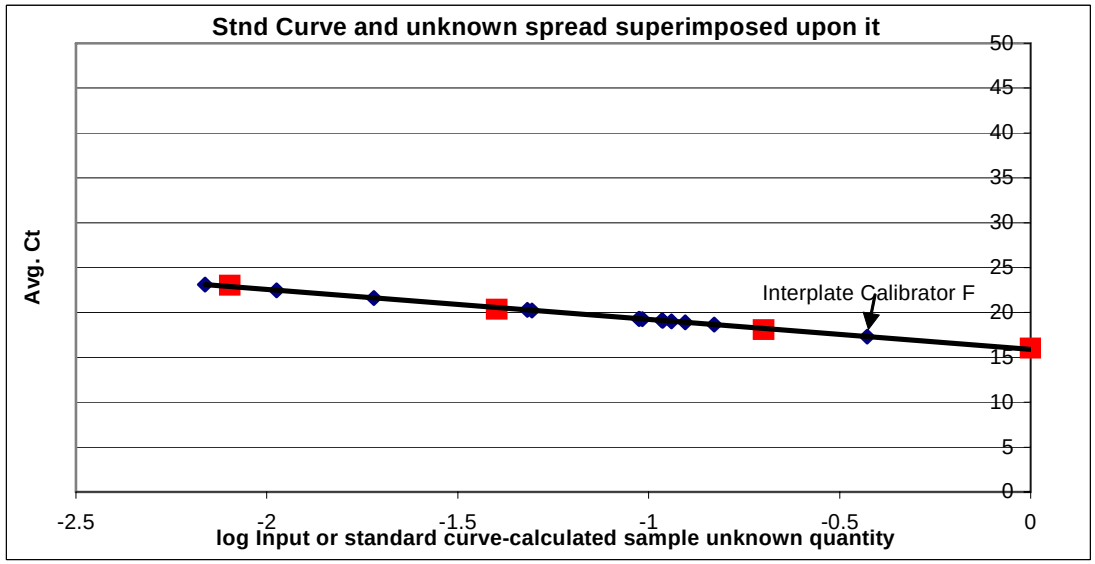
Notice the fine placement of the interplate calibrator – as predicted, and the acceptable spread of unknowns within the bounds of standard-curve linearity for all samples …

	G2 NTC		50.00
		Log of input or Q	Av. Ct
	Stnd1	0	23.61
	Stnd2	-1	26.68
	Stnd3	-2	30.16
	Stnd4	-3	32.90
0.217188	CALB	-0.663165	25.71
1.525971	14	0.183546	23.06
0.000416	15	-3.38138	34.24
5.77E-05	16	-4.238597	36.93
0.003648	17	-2.437905	31.28
0.004257	18	-2.37093	31.07
0.000413	19	-3.384209	34.25
0.001312	20	-2.882204	32.67
0.008986	21	-2.046413	30.05
0.001776	22	-2.750595	32.26
0.000344	23	-3.463762	34.53
0.00272	24	-2.565476	31.68
0.002473	25	-2.606693	31.82
0.003307	26	-2.480567	31.42



= standard curve points
= unknown samples

	18S NTC		39.37
		Log input or Qty	Av. Ct
	Stnd1	0	16.02
	Stnd2	-0.69897	18.10
	Stnd3	-1.39794	20.39
	Stnd4	-2.09691	23.04
0.373012	CALF	-0.428278	17.32
0.094563	14	-1.024278	19.31
0.019045	15	-1.720224	21.63
0.006895	16	-2.161464	23.10
0.096199	17	-1.016832	19.28
0.010612	18	-1.974221	22.48
0.094212	19	-1.025892	19.31
0.148541	20	-0.828155	18.65
0.124589	21	-0.90452	18.91
0.04808	22	-1.318032	20.29
0.11474	23	-0.940286	19.03
0.049421	24	-1.306089	20.25
0.108899	25	-0.962976	19.10
0.108168	26	-0.965899	19.11



Notice the fine placement of the interplate calibrator – as predicted, and the acceptable spread of unknowns within the bounds of standard-curve linearity for all samples …

ONE POTENTIALLY USEFUL AND INTERESTING DETAIL EMANANT OF THIS ENTIRE PROCEDURE IS THE OBSERVATION THAT CERTAIN TISSUE SAMPLE TYPES SEEM TO RESIST DNAse TREATMENT MORESO THAN OTHERS – AND THESE TISSUE SAMPLE TYPES ARE NOTORIOUSLY ALL HIGH IN COLLAGEN. DOES THIS INDICATE THAT COLLAGEN CAN INHIBIT DNAse ENZYMES? AND RT AND TAQ DNA POLYMERASE ENZYMES? AND COULD IT BE THAT CERTAIN qPCR STUDIES MAY INDEED BENEFIT FROM TREATMENT OF SAMPLES WITH COLLAGINASE(S) POST-ISOLATION IN THE EVENT THAT GENOMIC DNA CONTAMINATION OF TISSUE-SPECIFIC TOTAL RNA ISOLATES PRECLUDES THEIR USEABILITY AS REAL-TIME qPCR TEMPLATES?

BASED ON ACTUAL SUBTRACTION OF NRC Cts FROM TARGET Cts			
(G1 Avg.'d Cts)	Ct	(G2 Avg.'d Cts)	Ct
13	**14.415**	**Tongue1**	**14.56**
18	**14.89**	**Lung2**	**14.885**
16	**15.24**	**Crop2**	**14.99**
15	**16.71**	**Jej2**	**16.53**
24	**16.93**	**Trach2**	**16.915**
4	17.545	Testes1	17.635
26	17.81	Tongue2	17.84
22	17.91	Kidny2	17.715
8	17.99	Liver1	18.06
9	18.115	Kidny1	18.07
11	18.29	Trach1	18.38
3	18.32	Crop1	18.3
1	18.455	BoneM1	18.49
21	18.73	Liver2	18.675
7	18.785	Spleen1	18.82
5	18.82	Lung1	18.855
25	18.82	Conj2	18.79
20	18.94	Spleen2	18.85
10	19.04	Bursa1	19.075
17	19.05	Ovid2	19.02
19	19.19	Skin2	19.15
14	19.39	BoneM2	19.445
12	19.395	Conj1	19.415
6	19.695	Skin1	19.725
23	19.83	Bursa2	19.685
2	20	Jej1	19.955

How useful is this observation ?

ONE FINAL NOTE REGARDING **STOCK I**:

Recall that Stock I in this study was an equivolumetric mixture of the 26 total tissue RNA samples used in this study: just after their isolation by Trizol method, each RNA pellet was resolubilized in 150 μL of 0.1 mM EDTA pH 6.75, warmed to 65C for 5 minutes, and their spec. 260_{nm} and $260_{nm}/280_{nm}$ measurements at 1:50 were taken. 70 μL of each resolubilized RNA was then Turbo-DNAse treated, and 80 μL of each was then diluted 1:10 with nuclease-free water, and 50 μL of each RNA isolate was then mixed together into a single tube attaining a final volume of 1300 μL. This was our Stock I RNA solution from which all standards and interplate calibrators were prepared. It was also the mixture which served as the source of our serially-diluted template samples for the Test Plate we ran early on to identify the best RNA dilution ranges for each of 3 targets.

Each isolate thus comprised 1/26th of the final Stock I solution. And, as we have now noted in the aftermath of this study, the two Bone Marrow samples far outweighed all 24 other tissues with respect to their Gallinacin 1 (G1) and Gallinacin 2 (G2) content. The Bone Marrow RNA sample portion of the Stock I solution was therefore the 'most template-diluted' of all 26 samples in this regard – none of the other 24 samples contributed significantly to the overall concentration of G1 and G2 in the Stock I mixture … (in other words, if all samples were equal contributors, no single sample's G1-G2 template content would suffer dilution). Needless to say, the housekeeping target (Gallus gallus 18S rRNA) was abundant in all samples – as expected.

This observation becomes important as we realize the potential danger there is in blindly mixing samples together to create Stock I solutions [about which we have no choice but to do this] – for it is possible that only 1 of the contributing samples may contain the real-time target mRNAs of interest while the other samples serve only to dilute the signal strength of the Stock I solution in general. This could theoretically become a factor in identifying the useful RNA dilution ranges for each target's standard curve since serial Stock I dilutions studied on the Test Plate may on some occasions have already been compromised by the creation an "anemic" Stock I solution; i.e., one that contains very dilute target signals on account of it being a mixture of a preponderance of very weak contributors in the first place – where the strongest signal bearers of the lot have been muted. In the present case, the two Bone Marrow RNAs were such strong contributors to the Stock I solution's real-time qPCR G1 and G2 signals, that they superceded the potentially injurious paltry/diluting contributions made by the other 24 samples involved. On account of this, our Stock I was 'signal-worthy' enough to use with success here. All qPCR researchers would be well-advised to always include a strong positive qPCR-signal containing sample (or samples) among the lot they will study so that their "Stock I" solutions are valid and useful.

One result of using our particular Stock I solution is that we found that our Bone Marrow sample unknowns (of course) showed up in a region of each target standard curve that preceded the first standard in each case – since the first standard was prepared from Stock I. The dilution of the Bone Marrow samples themselves (according to what was discovered by the Test Plate for G1 and G2) to their useful 'first-tier' in-well concentrations of 0.03792 ng RNA/uL in each reaction well was ~3.5-times more concentrated (with respect to G1 and G2) than the useful Stock I-derived first point on the G1 and G2 standard curves. This illustrates why it is important to leave "wiggle room" (at both ends of each target or housekeeper standard curve) that still exhibits qPCR linearity at least ~2 to 5-fold above and ~2 to 5-fold below the highest and lowest dilution points of each standard curve (as also ascertained during Test Plate data analyses). This approach accommodates quite well the uncertainty that will invariably be associated with the blind-but-necessary creations of 'Stock I' solutions in general. Some researchers use plasmid-derived RNAs, or purified organismal DNA or RNAs as the source of template for their standard curves. However, this borders on idiocy at times when one's standard (and/or calibrator samples) are not prepared or derived in the exact same way as the experimental sample RNAs or DNAs are. Since unexpected qPCR inhibitory phenomena can vary by tissue, template source and template isolation method used, it is clearly best to employ the same approach to derive all templates used within the same real-time qPCR study – too many uncontrollable variables can be introduced otherwise.

The Bone Marrow samples in this study had relative G1 and G2 strengths of ~39.4 and 37.7, respectively, prior to their addition to the Stock I RNA mixture. Upon their addition to the Stock I mixture, we can calculate that their relative strength as G1 and G2 template contributors to the Stock I solution dropped to 1.51 and 1.45, respectively. All other tissue RNAs (with the possible yet still trivial exception of kidney and lung RNAs) contributed minimally (~4.5% collectively) to the overall G1 and G2 template content of the Stock I solution.

Fortunately, it is the nature of PCR to amplify extremely small amounts of starting nucleic acid template material, and this study benefited from this classic feature in that all tissue RNAs isolated for this study (and subsequently diluted appropriately on a target-by-target basis to optimal qPCR ranges) exhibited solid target signals. As a final note here: we contend that all Stock I solutions will be useful if they are comprised of the experimental samples involved in each qPCR study. The "Stock I" solution itself (by virtue of it being composed of either all the samples in a study, or those samples most expected to contain target) represents a self-mitigating/self-attenuating tool which is already tailored to the specific confines (known and unknown) of each particular qPCR study. A "closed system" is formed this way - a system that by default is allowed to establish its own perimeters and parameters by forcing it to use the very stuff it will study in order to study itself. ~jmg~

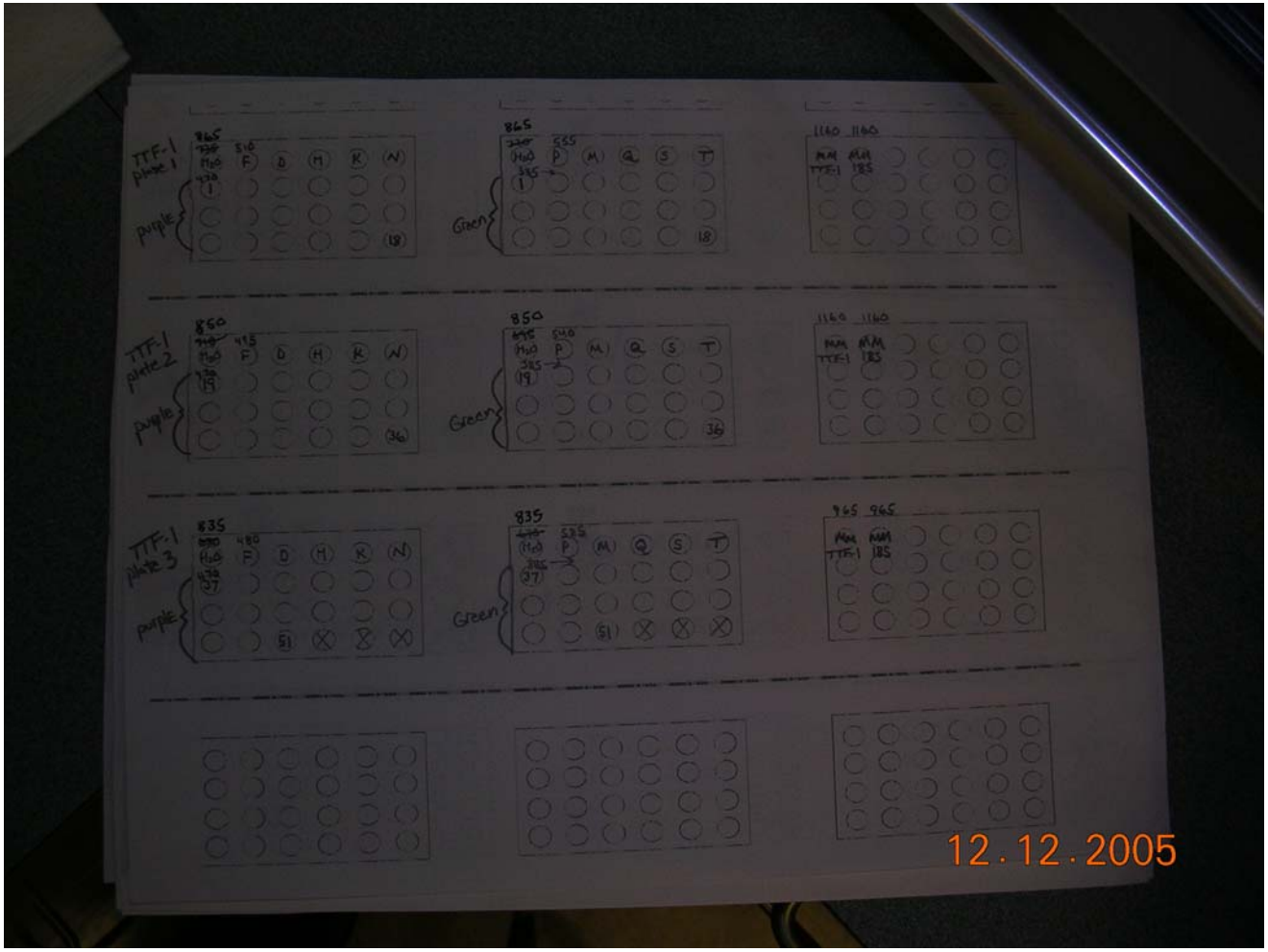
~PHOTOS~

PICTURE OF THE Eppendorf epMotion 5070 LIQUID HANDLING ROBOT

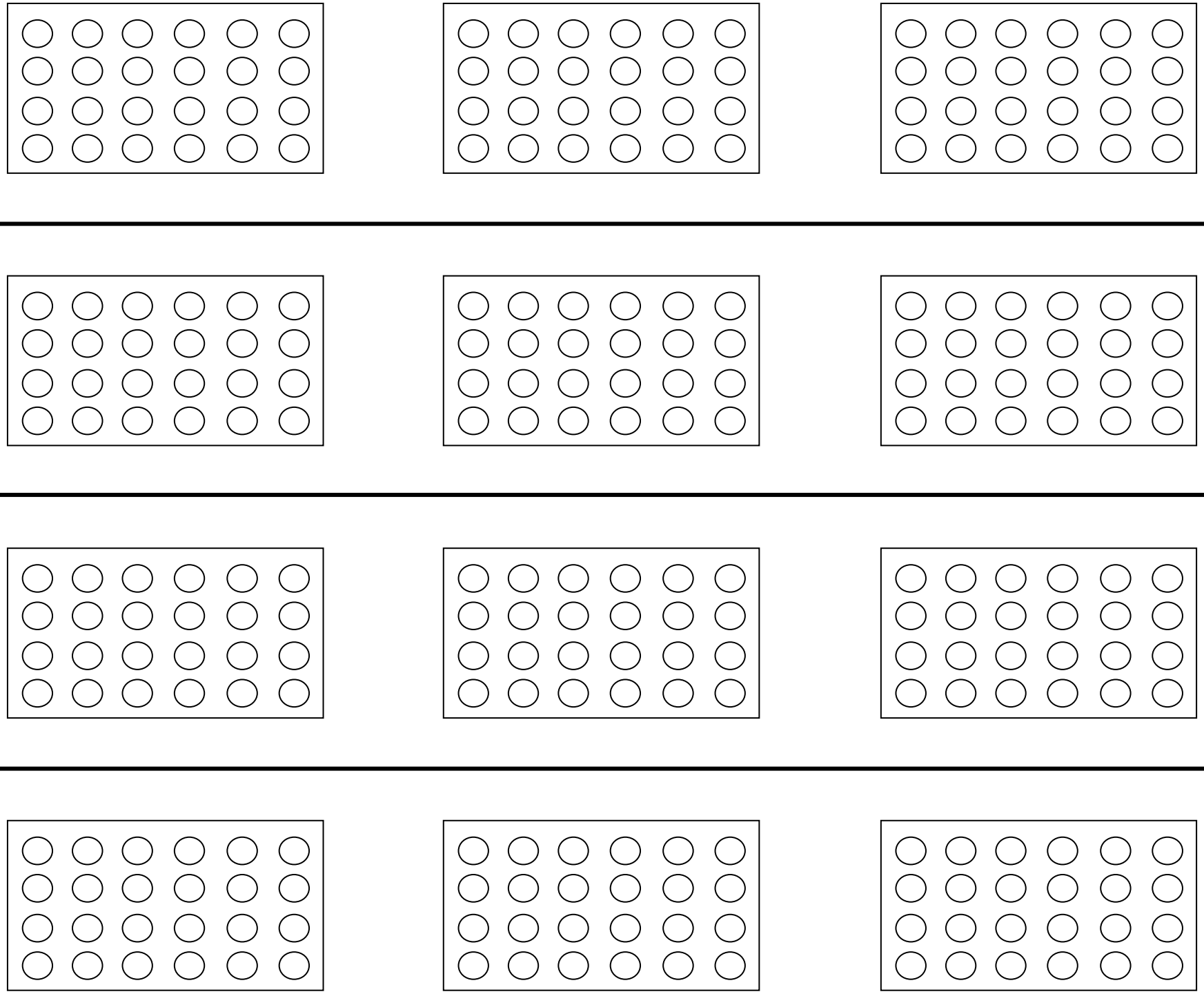
PICTURE OF A TYPICAL 1.5 mL TUBE SET-UP

PICTURE OF THE TUBES USED FOR MASTER MIX PREPARATIONS

PICTURE OF THE SHEETS WE USE TO DRAW OUT ROBOT PROCEDURES



(see next page for a better representation)



Sheet we created to draw out liquid-handling robot procedures

PICTURE OF THE Eppendorf epMotion 5070 ROBOT IN ACTION

PICTURE OF THE Eppendorf epMotion 5070 ROBOT IN ACTION

PICTURE OF TYPICAL STORAGE OF QPCR PLATES AT 4C BEFORE USE

PICTURE OF TYPICAL STORAGE OF QPCR PLATES AT 4C BEFORE USE

~ενδ~