PREPARATION OF SPIKING CONTROLS
Spiking Controls were from the AFGC Microarray Control Set.

I. Template Preparation
In order to efficiently transcribe spiking mRNAs which lack vector sequence and contain polyA tails, gene-specific primer pairs were designed with T3 promoter sequence fused to the 5'end of the FORWARD primer and with polyT sequence fused in the 3'end of the REVERSE primer.

Template: 1-10 ng DNA of Spiking Control (SP1-SP10) plasmid DNA
(or 5 ul of 1:25 dilution of mini-prep DNA)

Primers: will give 350-550 bp PCR product
SPxx-F contains T3 promoter at 5' end of 18-24-mer gene specific sequence
SPxx-R contains 21 polyT at 5' end of 18-24-mer gene specific sequence

Reaction Mix:

Reagent:	**Final Conc.**	**Vol. (µl)**
		X 1
Sterile filtered water		75.7
10X Promega PCR Buffer	1X	10
$MgCl_2$ (25mM)	1.5 mM	6
dNTP (20mM)	200 uM	1
SPx-F (20uM)	200 nM	1
SPx-R (20uM)	200 nM	1
Promega Taq Polymerase (5U/ul)	1.25 U	0.25
Pfu Polymerase (2.5U/ul)	0.125 U	0.05
Template		5

PCR Cycle**:**
1) 94º C for 2 min
2) 94º C for 30 sec
3) 57º C for 30 sec → (annealing temp will vary depending on primer pair Tm)
4) 72º C for 2.5 min
5) Go to step 2) for 30 X more
6) 72º C for 5 min
7) 4º C for ever
8) end

Resolve 5 ul of PCR mix on a 1.2% TAE agarose gel to check product.
Purify amplification products using QIAquick PCR Purification kit (QIAgen).
Determine DNA concentration using PicoGreen Assay or by A_{260} measurement.
Use speed vac to reduce volume to final concentration of 0.2-0.5 mg/ml DNA.

II. *In vitro* Transcription

Based on Promega RiboProbe in vitro Transcription System-T3 (P1430)

1. Add the following components at room temperature in the order listed:

Nuclease-free water	40 ul
5X Transcription Buffer	20 ul
DTT, 100 mM	10 ul
RNAse inhibitor (40U/ul)	2.5 ul (100 U)
rNTP (2.5 mM each)	20 ul (mix 1:1:1:1 of each 10 mM rNTP stock)
Template DNA	5 ul (1-2.5 ug)
T3 Polymerase (17U/ul)	2.5 ul (~40 U)
TOTAL	100 ul

(should yield 5-10 ug RNA/ug template DNA)

2. Incubate for 1-2 hours at 37-40 ^{o}C.
3. Remove 2 ul from this reaction for QC (Before-DNAse sample).

III. Removal of the Template DNA after Transcription

1. Add RQ1 RNase-Free DNAse to a concentration of 1U/ug template DNA.
2. Incubate for 15 minutes at 37 ^{o}C.
3. Remove 2 ul from this solution for QC (After-DNAse sample).
4. Purify RNA by using RNeasy Mini kit from QIAgen.
    a. Add 350 ul Buffer RLT to 100 ul RNA sample and mix thoroughly.
    b. Add 250 ul 95% ethanol to the diluted RNA and mix thoroughly by pipetting. Do not centrifuge. Continue immediately with step c.
    c. Apply the sample (~700 ul) to an RNeasy mini column placed in a 2 ml collection tube and centrifuge for 15 seconds at $\geq$ 8,000xg.
    d. Transfer the Rneasy column into a new 2 ml collection tube.
    e. Pipet 500 ul Buffer RPE onto the RNeasy column and centrifuge for 15 seconds at $\geq$ 8,000xg. Discard flow-through.
    f. Repeat step e.
    g. After discarding the flow-through recentrifuge RNeasy column at full speed for 1 minute.
    h. To elute, transfer the RNeasy column to a new 1.5 ml collection tube. Add 30-50 ul RNAse-free water directly onto the center of the RNeasy silica membrane. Incubate for 1 minute.
    i. Centrifuge for 1 minute at $\geq$ 8,000xg.
5. Quantify RNA by A_{260} measurement. Use 2 ul RNA + 48 ul TE per reading.
6. Add 1 ul RNAse inhibitor. Store at –20 ^{0}C.

Samples/OD	1	2	3	4	5
A260					
A280					
A260/A280					
Conc (ug/ml)					

IV. Test Quality of Spiking Control RNA

Add equal volume of 2X loading buffer to RNA sampled before and after DNAse treatment, heat at 70-80 ^{o}C for 3-5 minutes then resolve in 2% 1X TAE agarose gel with 1.0 ug/ml ethidium bromide. Use Low DNA Mass Ladder for quantitation of DNA and as markers. Run at 150 volts for 30 minutes. Take photo.

Steps:

1. Prepare 2% 1X TAE agarose gel with 1.0 ug/ml ethidium bromide:
    a. To 100ml 1X TAE add 2 g agarose. Melt completely in a microwave.
    b. Cool down under running water to about 60°C.
    c. Add 10 ul ethidium bromide (from 10 mg/ml stock)
    d. Pour the gel in a gel tray. Wait about 30 min to solidify.

2. Add equal volume of 2X loading buffer to QC samples. Put 1ul of original DNA (Sp1 and Sp3) as a control (+ 1ul 2X loading dye).

3. Load on the gel:
    Lane 1: Low DNA Mass Ladder (Invitrogen, cat # 10068-013) (6ul ladder + 6 ul 2X loading dye) for quantification.
    Lane 2: Original DNA for Sp1
    Lane 3: QC sample 1 for Sp1
    Lane 4: QC sample 2 for Sp1
    Lane 5: QC sample 3 for Sp1
    Lane 6: Original DNA for Sp3
    Lane 7: QC sample 1 for Sp3
    Lane 8: QC sample 2 for Sp3
    Lane 9: QC sample 3 for Sp3

3. Resolve the samples on the agarose gel (1XTAE running buffer).

4. Run at 150 volts for 30 min.

5. Take an unsaturated and a saturated pixels picture.

Spiking Controls
From AFGC

	GenBank #	ID	SPx-F Tm, ^{o}C	SPx-R Tm, ^{o}C
SP01	AF126021	B-cell receptor protein	49.2	51.4
SP02	X13988	Myosin Heavy Chain	61.0	53.3
SP03	M21812	Myosin Reg. Light Chain2	56.4	56.4
SP04	X07868	Insulin-like Growth Factor	51.6	49.2
SP05	AK001779	FLJ1091fis	54.0	56.9
SP06	AF161469	HSPC120	49.2	51.4
SP07	NM_004048	Beta2 microglobulin	N/A	N/A
SP08	NM_000291	Phosphoglycerate kinase (pgk1)	47.3	46.5
SP09	L11329	Tyrosine Phosphatase (pac-1)	60.8	61.9
SP10	U11861	G10 Homolog (edg-2)	58.2	61.0

Primers:

SP01-F
5'–CAA TTA ACC CTC ACT AAA GGG CGC GAA TCT GTG TTC ACC–3'

SP01-R
5'–TTT TTT TTT TTT TTT TTT TTT TAA TGG ACG GCA ACA CTC G–3'

SP02-F
5'–GCG CAA TTA ACC CTC ACT AAA GGG AGG AGC AGC TGG CGA TTG TGG–3'

SP02-R
5'–TTT TTT TTT TTT TTT TTT TTT CTC TGT GTT CTT CTT CTG CTC TCC–3'

SP03-F
5'–CAA TTA ACC CTC ACT AAA GGG TCG GGG AGA AGC TCA AGG G–3'

SP03-R
5'– TTT TTT TTT TTT TTT TTT TTT TAG GTC GGG CCG AAC AGA AG–3'

SP04-F
5'–GCG CAA TTA ACC CTC ACT AAA GGG GCA AGC GCC CTG TGA GT–3'

SP04-R
5'–TTT TTT TTT TTT TTT TTT TTT TGG GAT TGC AAG CGT TAC–3'

SP05-F
5'–GCG CAA TTA ACC CTC ACT AAA GGG GTG AAT CCT GAA ACA AAG AGA CCA–3'

SP05-R
5'–TTT TTT TTT TTT TTT TTT TTT GGC AGA CCA CAG ACA TGA AAC AGT–3'

SP06-F
5'–GCG CAA TTA ACC CTC ACT AAA GGG TTA CAG AAA GTT GGC GAT AGG–3'

SP06-R
5'–TTT TTT TTT TTT TTT TTT TTT AAC TCC CAT TGC AGA ACT TGA–3'

SP08-F
5'–GCG CAA TTA ACC CTC ACT AAA GGG TGG AAC ACG GAG GAT AAA G–3'

SP08-R
5'–TTT TTT TTT TTT TTT TTT TTT TGA GTA GTG AAA CAG TGA CAA AG–3'

SP09-F
5'–GCG CAA TTA ACC CTC ACT AAA GGG GGA GGC CGG GTG CTG GTG–3'

SP09-R
5'–TTT TTT TTT TTT TTT TTT TTT AAG GGC TTC AAC ATG GTG GTG GAC–3'

SP10-F
5'–GCG CAA TTA ACC CTC ACT AAA GGG AGG ATG GCT GGG AGT TGA TTG AG–3'

SP10-R
5'–TTT TTT TTT TTT TTT TTT TTT GAA GGG GGT GGC GTG ACA GG–3'