

## NARG 2005 Assay Information

The Nucleic Acid Research Group of the ABRF would like to thank you for agreeing to participate in this year's study. The assay kit contains reagents necessary for making two standard curves for a human b-Actin assay. A copy of this information is available at

[http://www.abrf.org/index.cfm/group.show/NucleicAcids.32.htm#R\\_4](http://www.abrf.org/index.cfm/group.show/NucleicAcids.32.htm#R_4).

The kit contains the following:

1. 1 nmole of Forward Primer: hb-Actin-997(+) (CCCTGGCACCCAGCAC).  
Dilute to 20  $\mu$ M by adding 50  $\mu$ l nuclease free water. Use at 400nM in your PCR reaction.
2. 1 nmole Reverse Primer: hb-Actin-1067(-) (GCCGATCCACACGGAGTAC)  
Dilute to 20  $\mu$ M by adding 50  $\mu$ l nuclease free water. Use at 400nM in your PCR reaction and in the RT reaction.
3. 0.4 nmoles Taqman® Probe: hb-Actin-1020(+) (FAM-ATCAAGATCATTGCTCCTCCTGAGCGCBHQ1)  
Dilute to 20  $\mu$ M by adding 20  $\mu$ l nuclease free water. Use at 100nM in your PCR reaction.
4. 400 pg synthetic DNA oligo template for the hb-Actin assay.  
Prepare a master stock at 20 pg/ $\mu$ l by adding 20  $\mu$ l nuclease free water as specified on the tube.  
Prepare a working stock at 2 pg/ $\mu$ l and then prepare 6 tenfold dilutions in the diluent provided.  
Use 4  $\mu$ l/reaction (0.8 pg in the highest dilution).
5. 400 pg *in vitro* transcribed RNA template for the hb-Actin assay.  
Prepare a master stock at 20 pg/ $\mu$ l by adding 20  $\mu$ l nuclease free water as specified on the tube. Prepare a working stock at 2 pg/ $\mu$ l and then prepare 6 tenfold dilutions in the diluent provided. Use 4  $\mu$ l/reaction (0.8 pg in the highest dilution).
6. 200  $\mu$ l of 100 ng/ $\mu$ l yeast tRNA in nuclease free water. Use as diluent for both templates.

Participants should use the RT and PCR chemistry(ies) and real-time hardware currently in use in their laboratories. The standard curves may be run with SYBR green I and without the probe if that is how you normally perform real-time PCR in your laboratory. There are enough reagents to run the standard curves two separate times, e.g., using both the Taqman® probe and SYBR green I or on two different platforms, or using two different Master Mixes. However, one set (RNA and DNA) of standard curves is sufficient for the study.

Treat both the DNA and RNA template dilutions as RNA. That is, run the RT reaction on both sets of standards even though there will be no cDNA synthesis in the DNA template dilution tubes. This is so the effect of the RTase on the *Taq* enzyme in the PCR is equal for both sets of standard dilutions. We request that labs that normally prime their RT reactions with oligo dT, random primers or a mixture of both use the reverse primer provided at 400 nM instead. This is because the RNA template is not long enough to allow many places for priming 3' to the start of the actual amplicon as would be the case in a full length b-Actin mRNA.

The final concentrations for the primers/probe are: 400 nM primers and 100 nM probe. The assays were initially designed utilizing a homemade master mix (see below) with 5 mM MgCl<sub>2</sub> and 200  $\mu$ M dNTPs. If you are using a kit, these concentrations may be determined already, which is fine.

**Be sure to use the included 100 ng/ $\mu$ l yeast tRNA-H<sub>2</sub>O solution to dilute the DNA and RNA standards. The carrier is important to ensure that the standards do not stick to the plastic labware**

**at the lowest concentrations.**

**Dilution of the DNA and RNA standards:**

- 1- The sRNA and sDNA master stocks should be at 100X (20 pg/ul).
- 2- The sRNA and sDNA working stocks should be at 10X (2 pg/ul).
- 3- Dilute the working stock sequentially in 10-fold decrements in the yeast tRNA-DEPC H<sub>2</sub>O provided.
- 4- One way is to add 3  $\mu$ l of standard to 27  $\mu$ l tRNA-H<sub>2</sub>O (see below).
- 5- Make a 6-log standard curve and run in duplicate. (12 wells/type of template).
- 6- Molecules for the sDNA start at 2E7 for 0.8 pg final.
- 7- Molecules for the sRNA start at 1E7 (longer template) for 0.8 pg final.
- 8- Curves should be parallel if not on top of each other.
- 9- Assays are set up for 4  $\mu$ l of standard/reaction. If you want less volume, compensate accordingly.

**Example:**

Standard No.	Stock (pg/ul)	Volume ul)	Diluent (ul)	Concentration (pg/ul)	Volume (ul)
1	2	3	27	0.2	30
2	0.2	3	27	0.02	30
3	0.02	3	27	0.002	30
4	0.002	3	27	0.0002	30
5	0.0002	3	27	0.00002	30
6	0.00002	3	27	0.000002	30

The following protocol is a 2-step procedure using a homemade master mix and a 50 µl PCR reaction. Use it as a guide for setting up the reactions only. If you are using a one-step or a 2-step kit, follow your normal procedure including the cycling conditions. Note, the RT reaction and PCR cycling conditions are a guide as well. This example is using a non-heat activated Taq and does not contain UNG. If you are using a kit, follow those instructions as per usual.

<b>RT-1</b>			1V	28V
[STOCK]	COMPONENT	FINAL CONC	VOLUME (µl)	VOLUME (µl)
*****	DEPC-H2O	*****	2.60	72.80
10X	Stratascript Bfr	1X	1.00	28.00
2.5 mM	dNTP Mix	500 µM	2.00	56.00
20 µM	hb-Actin-1067(-)	400 nM	0.20	5.60
50 U/µl	Stratascript	10 U/10µl	0.20	5.60
			6.00	168.00

- 1) ADD 6 µl RT-1 TO 24-WELLS
- 2) Add 4 µl hb-Actin sRNA to 2 RT-1 wells/dilution- Row A
- 3) Add 4 µl hb-Actin sDNA to 2 RT-1 wells/dilution- Row B
- 4) Put the plate in a thermocycler and run as follows:
  - 50°C - 30 min
  - 72°C - 5 min
  - 20°C - hold

<b>PCR-1</b>			1V	28V
[STOCK]	COMPONENT	FINAL CONC	VOLUME (µl)	VOLUME (µl)
*****	PCR-H2O	*****	23.00	644.00
10X	Invitrogen PCR Bfr	1X	5.00	140.00
100X	Invitrogen ROX	1X	0.50	14.00
50 mM	MgCl2	5 mM	5.00	140.00
2.5 mM	dNTP Mix	200 µM	4.00	112.00
20 µM	hb-Actin-997(+)	400 nM	1.00	28.00
20 µM	hb-Actin-1067(-)	400 nM	1.00	28.00
20 µM	hb-Actin-1020(+) <b>FAM</b>	100 nM	0.25	7.00
5 U/µl	Invitrogen Taq	1.25 U/50 µl	0.25	7.00
			40.00	1120.00

- 5) Add 40 µl PCR-1 to each well = 24
- 6) Run on a real-time machine as follows:
  - 95°C - 1 min
  - 40 cycles of **RAN on the 7700**
  - 95°C - 12 sec
  - 60°C - 30 sec