



Product Information

HPSA™ Assay Human β -actin mRNA Gene Expression Assay

Product Number **HK-3000**
Storage Temperature 2-8 °C

TECHNICAL BULLETIN

Product Description

The human β -actin ($h\beta$ -actin) messenger RNA (mRNA) Gene Expression Assay is a rapid DNA probe test that utilizes the Chromagen High Performance Signal Amplification (HPSA) technology for direct quantitation of $h\beta$ -actin mRNA in human cell samples.

PLEASE READ ALL INSTRUCTIONS THOROUGHLY BEFORE PERFORMING ASSAY

Beta-actin is a highly conserved cytoskeletal protein that is constitutively expressed at moderate levels in all eukaryotic cells.¹⁻⁴ Because expression of β -actin remains relatively constant under experimental conditions, it has been classified as one of the first "housekeeping genes" for which the mRNA may act as an internal standard

Nucleic acid hybridization tests are based on the efficient base-pairing of complementary nucleic acid strands to form stable double-stranded complexes. Following cell lysis and subsequent mRNA release, the mRNA is captured utilizing a 96-well plate coated with a covalently linked probe. Following hybridization of $h\beta$ -actin mRNA with a biotinylated single-stranded DNA probe, the captured $h\beta$ -actin probe complex is detected using streptavidin-labeled alkaline phosphatase and StarBright® Green substrate. Results are read on a microplate fluorometer equipped with an appropriate filter set.

Reagents Required

Product Description	Product Number	Fill Volume
$h\beta$ -actin Detection Probe	A 7476	1.1 mL
$h\beta$ -actin RNA Positive Control (20 attomoles/ μ l)	A 7726	0.15 mL
$h\beta$ -actin RNA Standard (50 attomoles/ μ l)	A 7601	0.45 mL
mRNA Black Capture Plate (96 well)	P 2994	1 each
mRNA Blocking Solution	B 3434	24 mL
Lyse-N-Hyb Reagent (dual purpose Reagent for both lysis & hybridization steps)	H 1537	14 mL
mRNA Wash Buffer (2 bottles)	W 4888	94 mL
mRNA Enzyme Reagent	E 7529	13 μ l
mRNA Enzyme Buffer	E 7654	14 mL
StarBright Green Substrate Reagent	G 4293	1 each
StarBright Substrate Diluent	D 5441	14 mL
mRNA Stop Buffer	S 0567	14 mL
mRNA Negative Control	N 6535	0.15 mL
Plate Sealer	S 1692	3 each
mRNA Standard Diluent	D 5316	1.4 mL

Materials Required but not Provided

- Phosphate Buffered Saline (Product No. D 8662)
- Disposable reagent reservoirs (Product No. R 9384)
- Disposable 1.5 mL tubes
- Disposable 15 mL tubes
- Centrifuge
- Vortex mixer
- 37 °C Incubator with plate shaker (See Procedural Notes for incubation recommendations that do not require a plate shaker)
- Fluorometer (See Table 1 for filter set recommendations)

Precautions and Disclaimers

Use only the coated 96 well black capture plate provided with the kit.

Do not mix reagents from different HPSA kits. The components of this kit are for Research Use only and not intended for diagnostic procedures.

Storage/Stability

For optimal performance, the unopened product should be stored in a dry place at 2 to 8 °C.

Refer to the Certificate of Analysis for expiration date. The Certificate of Analysis can be obtained from the Sigma-Aldrich website (www.sigma-aldrich.com).

Preparation Instructions

Equilibrate kit to room temperature (15-30 °C) for 20-30 minutes prior to use.

mRNA Blocking Solution (B 3434) and mRNA Wash Buffer (W 4888):

Warm the Wash Buffer and Blocking Solution at 37 °C for 10 minutes and mix by inversion. Equilibrate to room temperature (15-30 °C) and dissolve any precipitate which may have formed prior to use.

hβ-actin Standard (A 7601):

hβ-actin RNA standard is provided at a concentration of 50 attomoles/μl. Prepare four two-fold dilutions of the hβ-actin Standard in Standard Diluent (D 5316) to produce a suitable range of standards, e.g. 500, 250, 125, 62.5, 31.3 attomoles per well.

Standard Curve Preparation

1. Add 100 μl of hβ-actin Standard to a clean, dry microfuge tube labeled tube 1.
2. Add 50 μl of Standard Diluent to each of four additional microfuge tubes labeled tubes 2-5.
3. Transfer 50 μl from the first tube into the second tube and mix well by vortexing.
4. Create remaining serial dilutions by repeating step #3 for the remaining tubes labeled 2-5.

hβ-actin Detection Probe (A 7476):

Vortex or mix by inversion prior to use.

mRNA Enzyme Reagent (E 7529):

15-60 minutes prior to use add 5 μl of Enzyme Reagent to 0.5 ml of Enzyme Buffer (E 7654) and vortex. Prepare a working dilution by adding 5.5 μl of diluted enzyme to 11.0 ml Enzyme Buffer and place at room temperature until use.

NOTE: The final working concentration of the Enzyme Reagent is a 2 X 10⁵ dilution.

StarBright Green Substrate Reagent (G 4293):

Reconstitute the lyophilized Substrate Reagent by adding 12.0 ml of StarBright Substrate Diluent (D 5441). Thoroughly mix by vortexing. The reconstituted substrate may be stored at 2-8 °C for up to 2 weeks.

Sample Preparation

Adherent Cells

1. Remove tissue culture medium from adherent cells by aspiration.
2. Wash the cells with equal volume of PBS and remove by aspiration.
3. Lyse the cells to a concentration of 10⁶ cells per mL by adding the appropriate volume of Lyse-N-Hyb Reagent (H 1537).
4. Vortex or vigorously pipette the cells and buffer.
5. Incubate at room temperature for a minimum of 15 minutes.

Non-Adherent Cells

1. Centrifuge non-adherent cells at 500 X g for 5 minutes at 4 °C. Discard the supernatant.
2. Wash cells with one-half volume of PBS by gently pipetting to resuspend the cell pellet (DO NOT VORTEX to resuspend cell pellet).
3. Repeat step number 1.
4. Lyse the cells to a concentration of 10⁶ cells per mL by adding the appropriate volume of Lyse-N-Hyb Reagent (H 1537). Vortex or vigorously pipette the cells and buffer.
5. Incubate at room temperature for a minimum of 15 minutes.

The volume of Lyse-N-Hyb used may vary according to cell type and RNA content. For most cell types, lysates can be prepared at a concentration up to 2 x 10⁶ cells/mL.

Lysates are stable at ambient temperature (15 to 30 °C) for three days. For long term storage, lysates may be stored at -20 or -80 °C. Lysates can either be directly used in hybridization assays or further purified for isolation of total RNA or mRNA.

Procedural Notes

- When using induced cell lysates a baseline negative reference should be determined by including a sample of non-induced cells lysed in Lyse-N-Hyb Reagent. Pipet 100 μl of the lysate and 10 μl of Detection Probe into duplicate wells in

the Black Capture Plate. The results obtained from these non-induced sample values will correspond to the constitutive expression levels for the target gene.

- Cell lysate composition may effect the quantification of the mRNA. To determine this effect, add a known amount of standard mRNA to the lysate. Quantitate the concentration and compare to the baseline.
- If incubating without a plate shaker, extend the following incubation times:
Step 7: Extend hybridization to 2 hours
Step 13: Extend substrate incubation to 1.5 hours.
All other incubation periods remain at the indicated time.
- The h β -actin HPSA assay kit is suitable for use with robotics. For more information regarding this application, contact Sigma's Drug Discovery group at hts@sial.com

Assay Procedure

A standard curve MUST be determined each time the assay is performed. Standards should be assayed in duplicate.

Disposable pipet tips and reagent reservoirs should be used for all transfers to avoid cross-contamination of reagents or samples.

1. Block the plate by pipetting 200 μ l of Blocking Solution (B3434) into each well and incubating for 20 minutes at room temperature (15-30 $^{\circ}$ C).
2. Decant the Blocking Solution by quickly inverting the plate over a sink or receptacle and blot the plate twice on absorbent towels, making sure no liquid remains in the wells.
3. Add 10 μ l of Positive Control (A7726), 10 μ l of Negative Control (N6535) or 10 μ l of each standard in duplicate to the wells designated as controls or standards.
4. Add 90 μ l of Lyse-N-Hyb Reagent (H1537) to each well containing Positive Control, Negative Control or standard resulting in a total volume of 100 μ l/well.
5. Add 100 μ l of each sample cell lysate to the remaining wells of the assay plate.
6. Add 10 μ l of concentrated β -actin Detection Probe (A7476) to each well of the assay plate. The final hybridization volume for all wells is 110 μ l.
7. Cover the plate with a plate sealer, place on plate shaker, and incubate at 35-37 $^{\circ}$ C for 90 minutes.
8. Carefully remove plate sealer, quickly invert the plate over a sink or receptacle and blot the plate twice on absorbent towels making sure no liquid

remains in wells. Add 200 μ l of Wash Buffer (W4888) to each well. Pour off liquid as described above and repeat twice for a total of three washes.

9. Add 100 μ l of enzyme working dilution to each well.
10. Cover the plate with a fresh plate sealer, place on plate shaker, and incubate at 35-37 $^{\circ}$ C for 60 minutes.
11. Repeat step number 8.
12. Add 100 μ l of reconstituted StarBright Green Substrate to each well.
13. Cover the plate with a fresh Plate Sealer, place on plate shaker and incubate at 35-37 $^{\circ}$ C for 60 minutes.
14. Carefully remove the Plate Sealer and add 100 μ l of Stop Buffer (S0567) to each well to terminate the signal amplification.
15. Measure the fluorescence intensity of each well using a fluorometer equipped with an appropriate filter set. The optimum excitation and emission wavelengths for StarBright Green are 440 nm and 505 nm, respectively.
16. Cover the plate with a fresh Plate Sealer and store at room temperature or 2-8 $^{\circ}$ C. The fluorescent signal is stable at least 6 months when stored properly. Failure to properly seal the plate to prevent evaporation will compromise the stability of the signal.

Table 1: Commonly Used Plate Readers

Manufacturer	Model	Excitation Filter (peak/band dwidth)	Emission Filter (peak/band width)
Cambridge Technology	Fluorocount	440/20 nm	510/20 nm
Packard Instrument	Fluorocount	440/20 nm	510/20 nm
EG&G Wallac	Victor II (verify model)	445/30 nm	510/25 nm
Perkin Elmer	HTS 7000	450/35 nm	510/25 nm
Tecan	Spectrafluor	430 nm	535 nm
BMG Lab Technologies	Fluostar or Polarstar	440/10 nm	500 or 510/10 nm
Applied Biosystems	Cytofluor II or 4000	440/20 nm	508/20 nm
Molecular Devices	SpectraMAX	440 nm	505 nm
Thermo Labsystems	Fluoroskan Ascent	440/10 nm	510/10 nm

Standard fluorescein filters (485/535) may be used on most instruments, however, decreased signals may be observed. For additional information regarding a particular instrument or recommended filter set, please contact the manufacturer.

Results

The interpretation of results of the hβ-actin mRNA HPSA Assay is based on the signal of the sample expressed in Relative Fluorescence Units (RFU). The hbb-Actin mRNA HPSA Assay has been evaluated using the Fluoroskan Ascent FL (Thermo Labsystems, Helsinki, Finland) equipped with a 444/12 excitation filter and 510/10 emission filter.

The HPSA assays can detect mRNA in the low attomole range. Approximately 30 gene copies per cell can be detected. A typical standard curve is shown below. A standard curve must be generated for each set of samples assayed.

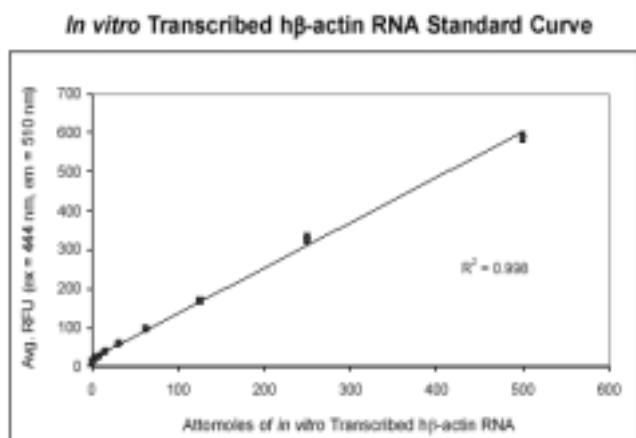


Figure 1: *In vitro* transcribed hβ-actin RNA standard curve.

Evaluation of Results

- Average the fluorescence readings for each standard or control. If high variability is observed for the standard or control, the assay should be repeated. Review the “Typical Assay Performance” table following this section for specific values.
- Construct a standard curve by plotting RNA quantity from the standard curve on the x-axis versus the fluorescence intensity (RFU) for the corresponding RNA quantity on the y-axis. The mean signal may be used.
- Results plotted as RFU vs. RNA quantity can be fit with a linear equation ($y = y_0 + ax$ where $y = \text{RFU}$; $y_0 = y$ intercept; $a = \text{slope}$; $x = \text{quantity of mRNA}$). Alternatively, plotting strategies including Power Fit, Logistic, and Log-Log may also be used.

- The quantity of β-actin mRNA in the unknown sample can be calculated by solving the regression equation for “x”. Note: If the samples have been diluted prior to testing, the concentration determined from the standard curve must be multiplied by the dilution factor.
- For accurate quantitation, the signal from unknown samples must be within the range covered by the standard curve. If signal of the sample exceeds the signal of the highest standard, further dilution of the sample is required. If signal of the sample is lower than that of the lowest standard, it may be considered a positive result if the value is greater than the mean of the negative control plus 2(Standard Deviation).

Typical Assay Performance

Working Range	0 – 500 attomoles/well
Detection Limit	≤ 31 attomoles/well
Within-Assay Precision	CV ≤ 20%
Positive Control Quantitation	≥ 100 attomoles
R ²	≥ 0.98

Assay performance may vary among commercial plate readers.

Performance characteristics of the hβ-actin mRNA HPSA Assay have been evaluated using RNA transcribed *in vitro* from cloned hβ-actin sequences. Assay performance was tested in constitutively expressed hβ-actin mRNA from two non-adherent cell lines (Hut 78 and THP-1 cells) and one adherent cell line (MG-63). Detection levels in the attomole range (10^{-18} mole) of *in vitro* transcribed RNA were routinely achieved using lysates from 10^5 cells per assay well.

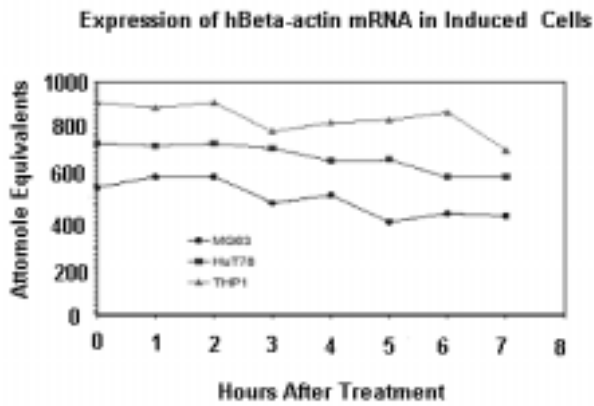



Figure 2: Expression of hBeta-actin mRNA in induced cells. Cells from three different immortal cell lines (GM-63, HuT 78, THP-1) were treated to induce expression of various cytokines. The cellular levels of hBeta-actin mRNA remain relatively constant, demonstrating its general utility as a transcriptional control.

References

1. Choi, J.K., et.al. 1991. Phorbol esters selectively and reversibly inhibit a subset of myofibrillar genes responsible for the ongoing differentiation program of chick skeletal myotubes. *Mol. Cell Biol.* **11**:4473-4482.
2. Thellin, O., et.al. 1999. Housekeeping genes as internal standards: use and limits. *J. Biotechnol.* **75**:291-295 cells per assay well.
3. Warrington, J.A., A. Nair, M. Mahadevappa, and M.Tsyganskaya. 2000. Comparison of human adult and fetal expression and identification of 535 housekeeping/maintenance genes. *Physiol. Genomics* **2**:143-147.

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Frequently Asked Questions

How long does it take to perform a typical HPSA assay?

The time needed to perform an HPSA Assay is approximately 4.5 hours (incubations require about 3.5 hours).
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Do I need to perform any preliminary purification on my samples or can I use cell lysates directly?
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The hIL-1 β HPSA Assay does not require further purification of the cell lysate prior to use.

What is the method of detection? Do I need special instrumentation to read the assay?
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The HPSA assay requires the use of a fluorescence plate reader. The excitation of StarBright Green is 440 nm and the emission is 505 nm, therefore, it is necessary to have compatible filter sets for optimal readings. Please refer to Table 1 for filter recommendations or contact the manufacturer of the instrument directly.

What if the incubation temperature recommended in the protocol is not followed?
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Allowing the temperature to fall too low compromises hybridization specificity which may dramatically alter observed sample signals. Conversely, if the temperature rises too high, hybridization/substrate digestion may be prevented resulting in low or absent signals. In general if any of the incubation temperatures fall outside the ranges given in the technical bulletin, it is best to consider the results suspect and repeat the assay.

What if the incubation time recommended in the protocol is not followed?

The Hybridization incubation (Step 7 of protocol) may be <u>extended</u> by 2 hours if needed. However, if the Hybridization incubation is <u>reduced</u> by greater than 10% there may not be sufficient annealing thereby altering the assay results.

Troubleshooting Criteria	Recommendations
High Background	<ul style="list-style-type: none"> The plate may not have been blocked or washed adequately. If plate washer is used, check output to be sure all wells are being washed/aspirated sufficiently. Substrate stock may have been contaminated during reconstitution/dilution. To verify, calculate background RFU's of a sample in a multiwell plate.
Low/No Signal	<ul style="list-style-type: none"> Verify the substrate reagent was reconstituted/diluted properly. Verify enzyme and or substrate were not added in the reverse order. Verify all appropriate reagents were added to the wells. Verify the 37°C incubators are functioning properly. If a plate washer is used, check that the system was primed with and operated with Wash Buffer only (if water was used, the hybrids will wash off and there will be no signal).
High Variability	<ul style="list-style-type: none"> Verify pipettors are calibrated and functioning properly. If cell lysates are viscous, vortex prior to pipetting into assay plate. If plate washer is used, check output to be sure all wells are being washed/aspirated to the same degree.
Edge Effects	<ul style="list-style-type: none"> Check that incubator is functioning properly. If a platform shaker is used, check to be sure the plates are uniformly shaking (especially important if plates are stacked on shaker). Verify the plastic sealer is properly secured to the plate, thereby minimizing any potential evaporation of reagents during incubation times. Some readers can confer edge effects. To validate the plate reader, read a plate twice, rotating 180° between reads, and compare the results (i.e. A1 = H12). Any reader effects can therefore be taken into account.
Sample out of range	<ul style="list-style-type: none"> If quantitation of sample is higher than the highest standard, dilute the sample and re-test. If quantitation of standard is lower than lowest standard, dilute standards such that samples will fall within the range of the standards.
Matrix Interference	<ul style="list-style-type: none"> Make sure that well washing is adequate. Verify concentration of cells prior to lysing. If target is expressed at a high level, dilute samples 1:2 or 1:4 and re-test. To compensate for the effect of matrix on samples, run standard curve in non-induced (target-negative) sample or matrix approximate prepared in Lyse-N-Hyb Reagent. Compare sample results to "spiked" standard curve.
Higher %CV for low abundant RNA samples and/or complex samples	<ul style="list-style-type: none"> Samples that contain low quantities of mRNA may have higher CV's both inter-assay and intra-assay due to the increased noise at the low end of the standard curve. The lower the RFU's, the more the precision of the instrument, pipetting variation, etc. contributes to the overall %CV of the sample. Be certain the induction time course has been optimized to maximize the expression of the target mRNA.

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