Quantikine® mRNA Probes and Calibrator Kit

Mouse iNOS

Catalog Number: RN951
Pack Size: 100 Tests
Lot Number:
Expiration Date:

Specifications and Use

Components

- Probes are supplied as a 6X concentrated stock solution (1.1 mL).
- RNA Calibrator is supplied as a 1200 attomole/mL (amol/mL) stock solution (1.1 mL).

RNA Calibrator Sequence and Size

- Genbank® Accession Number: NM_010927
- Cloned cDNA size: 3991 base pairs (bp)

Other Supplies Required

- Quantikine mRNA Base Kit (Catalog Number RN000).

Storage

- Store unopened kit at ≤ -70° C. Do not use past the expiration date above.
- **Avoid repeated freeze-thaw cycles.**

Instructions for Use

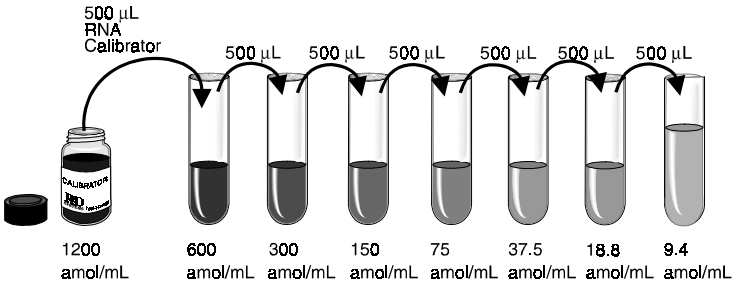
- Completely thaw reagents before use.
- Mix reagents by gentle inversion.
- Microcentrifuge briefly before use to prevent the loss of reagents.
- Refer to the Base Kit manual for the Quantikine mRNA assay procedure.

Preparation of Probes

- For 96 wells, add 1.0 mL of the probes to 5.0 mL of Sample Diluent (provided in the Quantikine mRNA Base Kit). For less than 96 wells, adjust volumes accordingly.
- Freeze the remaining undiluted probes at ≤ -70° C.
- Make a fresh dilution of probes before running each assay.

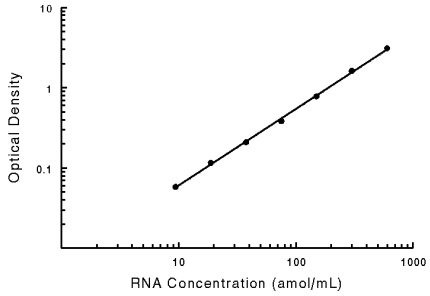
Preparation of RNA Calibrator

- Pipette 500 µL of Calibrator Diluent into each tube. Pipette 500 µL of RNA Calibrator into the 600 amol/mL tube. Continue the two-fold dilution series (shown below). Mix each tube thoroughly before each transfer. The 600 amol/mL calibrator serves as the high calibrator. The Calibrator Diluent serves as the zero calibrator.
- Freeze the remaining undiluted RNA Calibrator at ≤ -70° C.
- Make a fresh dilution of calibrators before running each assay.



Typical Data

This mouse iNOS calibrator curve is provided only for demonstration. A calibrator curve should be generated each time an assay is run.



amol/mL	O.D	Average	Corrected
0	0.098 0.104	0.101	-
9.4	0.156 0.161	0.159	0.058
18.8	0.216 0.217	0.217	0.116
37.5	0.307 0.312	0.310	0.209
75	0.477 0.493	0.485	0.384
150	0.855 0.911	0.883	0.782
300	1.620 1.823	1.722	1.621
600	3.252 3.158	3.205	3.104

R&D Systems Inc.
1-800-343-7475

Performance Characteristics

Sensitivity - Nine assays were evaluated and the minimum detectable dose (MDD) of mouse iNOS mRNA ranged from 2.1 - 4.0 amol/mL. The mean MDD was 2.8 amol/mL. The minimum detectable dose was determined by adding two standard deviations to the mean optical density value of 20 zero calibrator replicates and calculating the corresponding concentration.

Intra-assay Precision - Three samples of known concentration were assayed twenty times on one plate to assess precision within an assay.

Sample	1	2	3
n	20	20	20
Mean (amol/mL)	79.1	189	340
Standard Deviation	4.9	13.8	16.6
% CV	6.2	7.3	4.9

Inter-Assay Precision - Three samples of known concentration were assayed twenty times in separate assays to assess precision between assays.

Sample	1	2	3
n	20	20	20
Mean (amol/mL)	81.5	184	326
Standard Deviation	7.0	14.5	25.4
% CV	8.6	7.9	7.8

Sample Data - Raw 264.7 cells were stimulated with LPS (100 ng/mL) for 8 hours. Poly $(A)^+$ RNA was isolated using an Oligotex® mRNA Kit (Qiagen, Inc.).

Sample	Sample Amount (ng)	Expected amol/mL	Observed amol/mL
Stimulated Raw 264.7	500	-	562
	250	281	281
	125	140	152
	62.5	70.2	67.6
	31.3	35.1	34.4
	15.6	17.6	15.8
	7.8	8.8	9.9

Technical Hints:

- The amount of RNA needed per well is dependent on the sample type. For initial analysis, 5×10^5 to 2×10^6 cells/mL of Cell Lysis Diluent is recommended for cell lysate samples and 2 - 5 µg of total RNA per well is recommended for total RNA samples. Poly $(A)^+$ RNA samples may require less RNA than total RNA samples. The amount required for subsequent analyses can be adjusted after the target mRNA concentration is known. In sample types tested at R&D Systems, results were obtained using 250 - 2000 ng of total RNA and 7.8 - 500 ng of poly $(A)^+$ RNA.
- The approximate molecular weight for single stranded RNA (ssRNA) can be calculated by multiplying its length in nucleotides times the average mass per mole of RNA nucleotide (320 g/mol nucleotide).
- One attomole of mouse iNOS mRNA is approximately 1277 femtograms (1 g = 10^{15} fg). This conversion factor uses an estimated molecular weight for the ssRNA that is based on the cDNA size obtained from Genbank. The cDNA size does not include the cap structure or the poly (A) tail and may not include the entire untranslated regions.
- Expression of mouse iNOS mRNA is tightly regulated and elevated levels are transient.
- Mouse iNOS mRNA may not be detectable in unstimulated cells or in stimulated cells that were harvested when the mRNA was not near peak levels.

R&D Systems
614 McKinley Place NE
Minneapolis, MN 55413 USA

Quantikine® mRNA

BASE KIT

Catalog Number RN000

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

Quantitation of gene-specific mRNA is an important tool used routinely in the study of gene expression and function. Common methods include Northern blot (1), slot blot (2), reverse transcription followed by polymerase chain reaction (RT-PCR) (3), and ribonuclease protection assay (RPA) (4). Quantikine mRNA is a novel method which can be used to quantitate gene-specific mRNA at low levels. This assay is sensitive, specific and accurate without the known disadvantages of the traditional methods. Northern blot analysis allows semiquantitative determination of mRNA levels but is labor intensive, takes several days to perform, and is unsuitable for high sample throughput or for multiple mRNA analyses. Quantitative RT-PCR methods allow the greatest sensitivity (up to 1000-fold more sensitive than Northern blots) and are less susceptible to poor sample integrity; however, precise and accurate results may be difficult to obtain due to the exponential nature of PCR amplification. Furthermore, competitive RT-PCR methods are not amenable to high sample throughput and are dependent on a competitor that amplifies with the same efficiency as the target. RPAs allow mRNA quantitation with higher sensitivity (10 to 50-fold) and require less time than a Northern blot, but are labor intensive and typically require the use of a radioisotope.

Quantikine mRNA is a colorimetric microplate assay that can be completed in less than one day, while providing the sensitivity of a Northern blot. Adherent or non-adherent cells can be lysed using this kit. These cell lysates, total RNA, or poly $(A)^+$ RNA may be used in the assay. The Quantikine mRNA assay is amenable to high sample throughput and analysis of multiple mRNA targets on the same plate.

PRINCIPLE OF THE ASSAY

Samples are hybridized with gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes in a microplate. The hybridization solution is then transferred to a streptavidin-coated microplate and the RNA/probe hybrid is captured. Following a wash to remove unbound material, an anti-digoxigenin alkaline phosphatase conjugate is added. After washing away unbound conjugate, a substrate solution is added. An amplifier solution is then added and color develops in proportion to the amount of gene-specific mRNA in the original sample. Color development is stopped and the intensity of the color is measured spectrophotometrically.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Use ribonuclease-free (RNase-free) reagents and supplies when running this assay.
- The kit should not be used beyond the expiration date on the kit label.
- Any variation in diluents, operator, pipetting technique, washing technique, incubation time or temperature, or kit age can alter assay performance.

REAGENTS

Cell Lysis Diluent Concentrate (Part 895540) - 1.7 mL of a 10X concentrated solution.

Hybridization Plate (Part 895494) - One 96 well polystyrene microplate.

Streptavidin Plate (Part 890649) - One 96 well polystyrene microplate (12 strips of 8 wells) coated with streptavidin.

Calibrator Diluent (Part 895283) - 21 mL of a buffered protein solution with preservative.

Sample Diluent (Part 895284) - 21 mL of a buffered protein solution with preservative.

Anti-digoxigenin Conjugate (Part 890650) - 21 mL of polyclonal antibody against digoxigenin, conjugated to alkaline phosphatase, with preservatives.

Wash Buffer Concentrate (Part 895285) - 100 mL of a 10X concentrated solution with preservative.

Substrate (Part 895077) - Lyophilized NADPH with stabilizers.

Substrate Diluent (Part 895078) - 7 mL of a buffered solution with stabilizers.

Amplifier (Part 895075) - Lyophilized amplifier enzymes with stabilizers.

Amplifier Diluent (Part 895076) - 7 mL of a buffered solution containing INT-violet with stabilizers.

Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.

Plate Sealers - 12 adhesive strips.

Float Collar (Part 720045) - Microplate float collar for water bath.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="9">Opened/ Reconstituted Reagents</td><td>1X Wash Buffer</td><td rowspan="7">Store for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Calibrator Diluent</td></tr>
<tr><td>Sample Diluent</td></tr>
<tr><td>1X Cell Lysis Diluent</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>Substrate Solution</td><td rowspan="2">Store for up to 1 month at ≤ -20° C.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Amplifier Solution</td></tr>
<tr><td>Streptavidin Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack and reseal along entire edge of zip-seal. Store for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Quantikine mRNA gene-specific probes and calibrator kits supplied by R&D Systems.
- Microplate reader capable of measuring absorbance at 490 nm with the correction wavelength set at 650 nm or 690 nm.
- Pipettes and pipette tips.
- Deionized water, RNase-free.
- Multi-channel pipette, squirt bottle or manifold dispenser.
- Graduated cylinders: 100 mL and 1000 mL for preparation of Wash Buffer.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- 65 ± 1° C water bath.
- Vortex mixer.
- Gloves.

PRECAUTIONS

Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

TECHNICAL HINTS

- Avoid RNase contamination during reagent and sample preparation and while running the assay. The use of gloves, mask and a labcoat with tight-fitting cuffs is strongly recommended. RNase contamination will cause poor precision and/or depress signal in sporadic wells.
- Careful washing of the microplate is essential to minimize non-specific binding of the conjugate.
- Avoid contact of the conjugate with phosphate-based wash buffers and other sources of inorganic phosphate.
- Addition of the Substrate Solution or the Stop Solution will not result in a color change.
- Addition of the Amplifier Solution will result in a color change (colorless to shades of maroon).
- Amplifier Solution and Stop Solution should be added to the plate in the same order as the Substrate Solution.
- Warm reagents to room temperature and mix to homogeneity prior to pipetting.
- Avoid foaming when mixing reagents.
- To avoid cross-contamination, use a sterile pipet tip for each addition of calibrator, sample or reagent. Also, use separate reservoirs for each reagent.
- Proper adhesion of Plate Sealers during incubation steps is necessary.
- Cover plate with a new Plate Sealer before each incubation.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

1X Cell Lysis Diluent - Dilute 1.7 mL of 10X Cell Lysis Diluent Concentrate into 15.3 mL of Sample Diluent.

1X Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 100 mL of 10X Wash Buffer Concentrate into 900 mL of deionized water to prepare 1000 mL of Wash Buffer.

Substrate Solution - Dissolve the lyophilized Substrate in 6 mL of Substrate Diluent at least 10 minutes before use. Mix thoroughly. **Discard the rubber stopper after reconstitution**.

Amplifier Solution - Dissolve the lyophilized Amplifier in 6 mL of Amplifier Diluent at least 10 minutes before use. Mix thoroughly. **Discard the rubber stopper after reconstitution**.

ASSAY TEMPLATE

Multiple gene-specific targets can be tested on the same microplate. The format below shows sixteen samples (A-P) tested for two targets. This template is for demonstration purposes only and the layout can be varied to fit each assay. A blank template is provided on page 11.

C = Calibrator SMP = Sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	SMP A	SMP A	SMP I	SMP I	0	0	SMP A	SMP A	SMP I	SMP I
B	C1	C1	SMP B	SMP B	SMP J	SMP J	C1	C1	SMP B	SMP B	SMP J	SMP J
C	C2	C2	SMP C	SMP C	SMP K	SMP K	C2	C2	SMP C	SMP C	SMP K	SMP K
D	C3	C3	SMP D	SMP D	SMP L	SMP L	C3	C3	SMP D	SMP D	SMP L	SMP L
E	C4	C4	SMP E	SMP E	SMP M	SMP M	C4	C4	SMP E	SMP E	SMP M	SMP M
F	C5	C5	SMP F	SMP F	SMP N	SMP N	C5	C5	SMP F	SMP F	SMP N	SMP N
G	C6	C6	SMP G	SMP G	SMP O	SMP O	C6	C6	SMP G	SMP G	SMP O	SMP O
H	C7	C7	SMP H	SMP H	SMP P	SMP P	C7	C7	SMP H	SMP H	SMP P	SMP P

TARGET 1 TARGET 2

SAMPLE PREPARATION

The same sample preparation method should be used for all samples because values obtained for samples prepared by different methods may not be comparable.

A. CELL LYSATE SAMPLES

Cell lysates can be made from fresh or frozen cells by adding 1X Cell Lysis Diluent to the cell samples. Excess media should be removed from cell pellets or monolayers; however, washing with a buffered saline solution is not necessary. Cell lysates can be prepared from fresh cells and frozen for use at a later time. Avoid multiple freeze-thaw cycles for both cell pellets and cell lysate samples. The cell lysate procedure has not been validated for use with tissue samples.

Prepare cell lysate samples using the following procedure:

1. Add 1X Cell Lysis Diluent to obtain a final concentration of 5×10^5 - 2×10^6 cells/mL. This concentration range is recommended for both adherent and non-adherent cells. Adherent cells can be lysed directly in the flask after removal of media. Frozen cell pellets should be kept on dry ice until ready to use.

2. Pipet the cells up and down several times until they are resuspended.

3. Vortex the lysed cells for 15 to 20 seconds.

4. Add 150 µL of the cell lysate sample to the respective wells as described in step 4 of the Assay Procedure. Cell lysate samples may need to be diluted in 1X Cell Lysis Diluent to obtain a value in the range of the calibrator curve. Cell lysate samples should be used promptly in the assay or immediately frozen. Frozen cell lysates should be thawed on ice prior to use.

B. RNA SAMPLES

Total RNA and poly $(A)^+$ RNA are suitable samples for the Quantikine mRNA assay. We recommend purification of total RNA using the Qiagen RNeasy® Maxi Kit. For the purification of poly $(A)^+$ RNA, we recommend using the Qiagen Oligotex® mRNA Midi Kit. The same purification method should be used for all RNA samples because values obtained for RNAs purified by different methods may not be comparable.

The concentration of RNA samples should be determined at A_{260} using absorbance values in the linear range of the spectrophotometer, typically 0.1 - 1.0. An optical density of 1.0 at A_{260} is equal to an RNA concentration of 40 µg/mL. The A_{260}/A_{280} ratio for RNA samples should be 1.8 - 2.2 (5). Aliquot and store RNA samples at ≤ -70° C. Avoid multiple freeze-thaw cycles.

The amount of RNA required per well is dependent on the target mRNA abundance. Refer to the gene-specific Probes and Calibrator Kit specification sheet for an approximate range. An example of a sample preparation for analysis of 2 µg of RNA per well follows: Add 5.2 µL of an RNA sample (0.9 µg/µL) to 345 µL of Sample Diluent, then use 150 µL diluted sample per well. This sample dilution will provide sufficient volume to perform the assay in duplicate and provide excess for pipetting. The volume of sample RNA stock solution used per well should not exceed 15 µL.

ASSAY PROCEDURE

Wear gloves, mask, and a labcoat with tight-fitting cuffs during all assay steps. Bring all reagents to room temperature before use. Thaw samples on ice. Assaying all samples and calibrators in duplicate is recommended.

1. ❒ Prepare reagents and samples as instructed. Refer to the gene-specific specification sheet for instructions on preparation of probes and calibrators.
2. ❒ Wash the Hybridization Plate 2 times with Wash Buffer. Remove excess Wash Buffer by decanting or aspirating. Invert the plate and blot against clean paper towels.
3. ❒ Add 50 µL of gene-specific probes to the designated wells.
4. ❒ Add 150 µL of Calibrator or sample to the designated wells. Cover with a Plate Sealer.
5. ❒ Apply the Float Collar to the Hybridization Plate and incubate the plate for 60 minutes in a 65° C water bath.
6. ❒ Remove unused microplate strips from the Streptavidin Plate frame, return them to the foil pouch containing the desiccant pack, reseal.
7. ❒ Wash the Streptavidin Plate 2 times with Wash Buffer and remove excess Wash Buffer as described in step 2.
8. ❒ Transfer 150 µL from each well of the Hybridization Plate to the washed Streptavidin Plate and apply a new Plate Sealer.
9. ❒ Incubate for 60 minutes at room temperature (20 - 25° C) on a shaker set at 500 ± 50 rpm.
10. ❒ Wash the Streptavidin Plate 4 times with Wash Buffer and remove excess Wash Buffer.
11. ❒ Add 200 µL of Anti-digoxigenin Conjugate to each well and cover with a new Plate Sealer.
12. ❒ Incubate for 60 minutes on a shaker at room temperature.
13. ❒ Wash the Streptavidin Plate 6 times with Wash Buffer and remove excess Wash Buffer.
14. ❒ Add 50 µL of Substrate Solution to each well and cover with a new Plate Sealer.
15. ❒ Incubate for 60 minutes on a shaker at room temperature. **Do not wash**.
16. ❒ Add 50 µL of Amplifier Solution to each well and cover with a new Plate Sealer.
17. ❒ Incubate for 30 minutes on a shaker at room temperature. **Do not wash**.
18. ❒ Add 50 µL of Stop Solution to each well.
19. ❒ Determine the optical density of each well within 30 minutes, using a microplate reader set to 490 nm. If wavelength correction is available, set to 650 nm or 690 nm.
    If wavelength correction is not available, subtract readings at 650 nm or 690 nm from the readings at 490 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 490 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

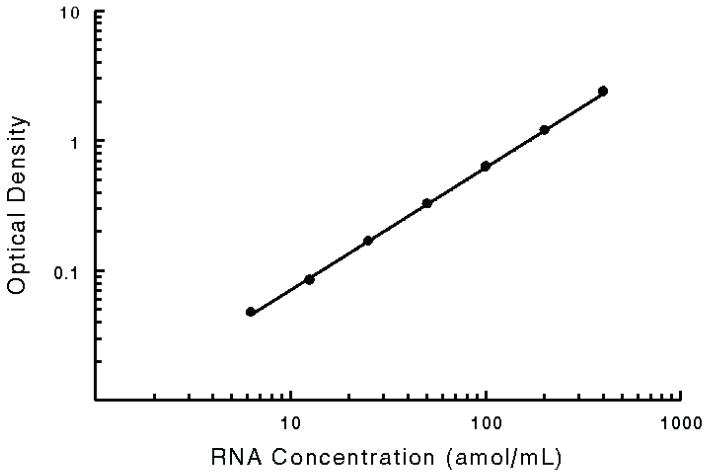
Average the duplicate readings for each calibrator and sample and subtract the average zero calibrator optical density. Graph the optical density versus the concentration of the calibrators and plot using a log/log curve fit.

To determine the concentration of mRNA in each sample, first find the optical density on the y-axis and extend a horizontal line to the calibrator curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration. Multiply results by the dilution factor.

Conversion Factor:
1 attomole or amol (10^{-18} mole) ≅ 600,000 molecules

EXAMPLE CALIBRATOR CURVE

The following calibrator curve is provided only for demonstration. A calibrator curve should be generated each time an assay is run.



amol/mL	O.D.	Average	Corrected
0	0.092 0.093	0.092	--
6.25	0.138 0.141	0.140	0.048
12.5	0.179 0.175	0.177	0.085
25	0.263 0.261	0.262	0.170
50	0.421 0.418	0.420	0.328
100	0.737 0.718	0.728	0.636
200	1.316 1.294	1.305	1.213
400	2.504 2.485	2.494	2.402

TROUBLESHOOTING GUIDE

Observation	Problem	Corrective Action
High background level	Insufficient washing	Wash per protocol being sure to remove all Wash Buffer from wells before addition of next component
	Contamination with alkaline phosphatase	Keep work area clean and free of alkaline phosphatase
Poor precision (high coefficient of variation) for samples or calibrator	Plate not washed before use	Wash per protocol
	RNase contamination	Use RNase-free technique
	Pipetting error	Use a new pipet tip for each pipetting step and use proper technique
No signal for calibrator curve	Component or step omitted	Read protocol thoroughly before repeating the assay
	RNase contamination	Use RNase-free technique
Sample signal is above calibrator curve range	Too much sample used	Repeat assay using less sample
Sample signal is below calibrators curve range, but calibrator curve works	Samples may be degraded	Run RNA gel to assess sample integrity
	Inadequate amount of sample used	Repeat assay using more sample
	Samples do not contain target mRNA	Verify that cell source was harvested near peak level of target mRNA

REFERENCES

1. Sagerstrom, C.G. and H. Sive (1996) *RNA Blot Analysis.* In *A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis.* P.A. Krieg ed., Wiley-Liss, N.Y., p. 83.
2. Brown, T. and K. Mackey (1997) *Analysis of RNA by Northern and Slot blot hybridization.* In *Current Protocols in Molecular Biology.* Ausubel, F.M. *et al*. eds., Wiley-Liss, N.Y., p. 4.9.1.
3. Ferré, F. *et al*. (1996) *Quantitation of RNA Transcripts Using RT-PCR*. In *A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis.* P.A. Krieg ed., Wiley-Liss, N.Y., p. 175.
4. Goldrick, M. *et al*. (1996) *Analysis by Nuclease Protection*. In *A Laboratory Guide to RNA: Isolation, Analysis, and Synthesis.* P.A. Krieg ed., Wiley-Liss, N.Y., p. 105.
5. Sambrook, J. and D.W. Russell (2001) *Quantitation of Nucleic Acids*. In *Molecular Cloning: A Laboratory Manual.* 3rd ed. Cold Spring Harbor Laboratory Press, N.Y., p. A8.19

APPLICABLE PATENTS

These products are covered by the following patents:

Substrate (Part 895077) - Lyophilized NADPH with stabilizers.

Substrate Diluent (Part 895078) - 7 mL of buffered solution with stabilizers.

Amplifier (Part 895075) - Lyophilized amplifier enzymes with stabilizers.

Amplifier Diluent (Part 895076) - 7 mL of buffered solution containing INT-violet with stabilizers.

US:	4,446,231 4,595,655 4,598,042
EUROPE:	60,123 27,036
CANADA:	1,170,179
AUSTRALIA:	544,496

ASSAY RECORD

This template may be used as a record of calibrators and samples run in an assay.