applied biosystems
by life technologies

TaqMan® Protein Assays

Probe Development Protocol

For Research Use Only. Not intended for any animal or human therapeutic or diagnostic use.

Information in this document is subject to change without notice.

APPLIED BIOSYSTEMS DISCLAIMS ALL WARRANTIES WITH RESPECT TO THIS DOCUMENT, EXPRESSED OR IMPLIED, INCLUDING BUT NOT LIMITED TO THOSE OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. TO THE FULLEST EXTENT ALLOWED BY LAW, IN NO EVENT SHALL APPLIED BIOSYSTEMS BE LIABLE, WHETHER IN CONTRACT, TORT, WARRANTY, OR UNDER ANY STATUTE OR ON ANY OTHER BASIS FOR SPECIAL, INCIDENTAL, INDIRECT, PUNITIVE, MULTIPLE OR CONSEQUENTIAL DAMAGES IN CONNECTION WITH OR ARISING FROM THIS DOCUMENT, INCLUDING BUT NOT LIMITED TO THE USE THEREOF, WHETHER OR NOT FORESEEABLE AND WHETHER OR NOT APPLIED BIOSYSTEMS IS ADVISED OF THE POSSIBILITY OF SUCH DAMAGES.

NOTICE TO PURCHASER: LIMITED LICENSE

Limited Use Label License: Research Use Only

The purchase of this product conveys to the purchaser the limited, non-transferable right to use the purchased amount of the product only to perform internal research for the sole benefit of the purchaser. No right to resell this product or any of its components is conveyed expressly, by implication, or by estoppel. This product is for internal research purposes only and is not for use in commercial applications of any kind, including, without limitation, quality control and commercial services such as reporting the results of purchaser's activities for a fee or other form of consideration. For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

Limited Use Label License: Product Disclaimer

The products referred to in this Protocol may be covered by one or more Limited Use Label License(s). Please refer to the respective product documentation or the Applied Biosystems website under www.appliedbiosystems.com for the comprehensive license information. By use of these products, the purchaser accepts the terms and conditions of all applicable Limited Use Label Licenses. These products are sold for research use only, and are not intended for human or animal diagnostic or therapeutic uses unless otherwise specifically indicated in the applicable product documentation or the respective Limited Use Label License(s). For information on obtaining additional rights, please contact outlicensing@lifetech.com or Out Licensing, Life Technologies, 5791 Van Allen Way, Carlsbad, California 92008.

TRADEMARKS

The trademarks mentioned herein are the property of Life Technologies Corporation or their respective owners.

TaqMan is a registered trademark of Roche Molecular Systems, Inc.

Excel and Microsoft are registered trademarks of Microsoft Corporation in the United States and other countries.

EZ-Link, Micro BCA, No-Weigh, and Slide-A-Lyzer are trademarks of Thermo Fisher Scientific.

Part Number 4449282 Rev. C
02/2011

Contents

About This Guide

Safety information

For general safety information, see this section and Appendix E, "Safety" on page 55.

Safety alert words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below:

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

CAUTION! – Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

WARNING! – Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

DANGER! – Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

SDSs

The Safety Data Sheets (SDSs) for any chemicals supplied by Applied Biosystems or Ambion are available to you free 24 hours a day. For instructions on obtaining SDSs, see "SDSs" on page 56.

IMPORTANT! For the SDSs of chemicals not distributed by Applied Biosystems or Ambion contact the chemical manufacturer.

TaqMan® Protein Assays Probe Development Protocol

Product information

Purpose of the product

Used in conjunction, the following kits enable you to develop Assay Probes from your own biotinylated antibodies:

- TaqMan® Protein Assays Oligo Probe Kit
- TaqMan® Protein Assays Buffer Kit
- TaqMan® Protein Assays Core Reagents Base Kit
- TaqMan® Protein Assays Fast Master Mix

After developing the Assay Probes, you can use them in TaqMan® Protein Assays experiments. The TaqMan® Protein Assays reagents enable detection and relative quantitation of proteins in cultured mammalian cell and tissue lysates.

For more information

For information on:

- Performing TaqMan® Protein Assays experiments, refer to the *TaqMan® Protein Assays Sample Prep and Assay Protocol*.
- TaqMan® Protein Assays technology, refer to the *Real-Time PCR Systems TaqMan® Protein Assays Chemistry Guide*.

See "Documentation" on page 59.

Kit contents and storage

Probe kits

Kit	Part number		No. of reactions	Contents	Storage conditions
	Kit	Top-fill†			
TaqMan® Protein Assays Oligo Probe Kit	4448549	4453745	4000	3′ Prox-Oligo, 200 nM (50 µL)	4°C
				5′ Prox-Oligo, 200 nM (50 µL)	

† Top-fill part numbers include both the probe and buffer kits.

Buffer kits

Kit	Part number		No. of reactions	Contents	Storage conditions	
	Kit	Top-fill†			Long-term	Short-term
TaqMan® Protein Assays Buffer Kit	4448571	4453745	4000	Antibody Dilution Buffer (1 mL) Assay Probe Storage Buffer (1 mL) Assay Probe Dilution Buffer (7 mL) Lysate Dilution Buffer (25 mL)	–15 to –25°C	After initial use, store the **Assay Probe Dilution Buffer** and **Lysate Dilution Buffer** at 2 to 8°C for up to 3 months.

† Top-fill part numbers include both the probe and buffer kits.

Core reagents base kits

Kit	Part number		No. of reactions	Contents	Storage conditions	
	Kit	Top-fill†			Long-term	Short-term
TaqMan® Protein Assays Core Reagents Base Kit (100 rxn)	4405460	4405501	100	DNA Ligase, 500× (10 µL) Ligase Dilution Buffer, 1× (2 × 1.5 mL) Ligation Reaction Buffer, 20× (0.7 mL) 1× PBS, pH 7.4 (1 mL) (Phosphate Buffered Saline) Protease, 100× (10 µL) Universal PCR Assay, 20× (120 µL)	–15 to –25°C	After initial use, store the **1× PBS** at 2 to 8°C for up to 3 months.

Kit	Part number		No. of reactions	Contents	Storage conditions	
	Kit	Top-fill†			Long-term	Short-term
TaqMan® Protein Assays Core Reagents Base Kit (500 rxn)	4448592	4448591	500	DNA Ligase, 500× (50 µL)	–15 to –25°C	After initial use, store the **1× PBS** at 2 to 8°C for up to 3 months.
				Ligase Dilution Buffer, 1× (15 mL)		
				Ligation Reaction Buffer, 20× (4 mL)		
				1× PBS, pH 7.4 (5 mL) (Phosphate Buffered Saline)		
				Protease, 100× (50 µL)		
				Universal PCR Assay, 20× (600 µL)		

† Top-fill part numbers include both the core reagents base kits and master mix.

Master mix

Kit	Part number		No. of reactions	Contents	Storage conditions
	Kit	Top-fill†			
TaqMan® Protein Assays Fast Master Mix, 2× (100 rxn)	4400088	4405501	100	Fast Master Mix, 2× (1.2 mL)	4°C
TaqMan® Protein Assays Fast Master Mix, 2× (500 rxn)	4448616	4448591	500	Fast Master Mix, 2× (6 mL)	4°C

† Top-fill part numbers include both the core reagents base kits and master mix.

User-supplied materials

The materials listed in this section are required to perform the procedures in this protocol, but are not included in the TaqMan® Protein Assays kits. Unless otherwise indicated, all items are available from major laboratory suppliers (MLS).

Biotinylated antibody

Prepare the Assay Probes with biotinylated antibody. See "Guidelines for selecting biotinylated antibodies" on page 13.

IMPORTANT! You must use biotinylated antibodies to prepare the Assay Probes. If the antibody you select is not biotinylated, you will need additional materials to label the non-biotinylated antibody with biotin. See Appendix B, "Select and Prepare Non-Biotinylated Antibodies" on page 39.

General laboratory equipment

Item	Source
Disposable gloves	MLS
Pipette tips (aerosol-resistant)	MLS
Pipettes (positive/air-displacement or multichannel)	MLS
Polypropylene tubes (various sizes)	MLS
For storing antibody that has passed the Forced Proximity Probe Test:	
• NALGENE 2-mL tubes or 0.5-mL tubes	Thermo Fisher Scientific (PN 342800-0020 or PN 342800-0005)
• NALGENE caps	Thermo Fisher Scientific (PN 342820-0110)
Vortexer	MLS
Centrifuge (with plate adapter)	MLS
Microcentrifuge	MLS
For all incubation steps: Thermal cycler with a heated cover **Note:** Applied Biosystems recommends using a thermal cycler for all incubation steps; however, you can use an incubator for the binding and ligation incubation steps.	Applied Biosystems GeneAmp® PCR System 9700, or equivalent
For use with the thermal cycler during the incubation steps: • MicroAmp® Clear Adhesive Film • MicroAmp® Optical Film Compression Pad, 5 pads	 • Applied Biosystems (PN 4306311) • Applied Biosystems (PN 4312639)

Reaction plates and accessories

The table below lists the reaction plates and accessories available for Applied Biosystems real-time PCR systems.

Real-time PCR system (Fast system recommended)	Reaction plates and accessories
7500 Fast system	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971) • MicroAmp® Optical 8-Cap Strip, 300 strips (PN 4323032)
7900HT/7900HT Fast system, Standard 96-Well Block Module	• MicroAmp® Optical 96-Well Reaction Plate with Barcode: – 500 plates (PN 4326659) – 20 plates (PN 4306737) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971) • MicroAmp® Optical Film Compression Pad, 5 pads (PN 4312639) • MicroAmp® Optical 8-Cap Strip, 300 strips (PN 4323032) • MicroAmp® Snap-On Optical Film Compression Pad, for use with the automation accessory (PN 4333292)
7900HT/7900HT Fast system, 384-Well Block Module	• MicroAmp® Optical 384-Well Reaction Plate with Barcode: – 1000 plates (PN 4343814) – 500 plates (PN 4326270) – 50 plates (PN 4309849) • MicroAmp® Optical 384-Well Reaction Plate, 1000 plates (PN 4343370) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971)
7900HT/7900HT Fast system, Fast 96-Well Block Module	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971) • MicroAmp® Snap-On Optical Film Compression Pad, for use with the automation accessory (PN 4333292)
StepOnePlus™ system	• MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode: – 200 plates (PN 4366932) – 20 plates (PN 4346906) • MicroAmp® Optical Adhesive Film, 100 films (PN 4311971)

Workflow

Select and prepare biotinylated antibodies (page 13)

(user-supplied biotinylated antibody)

↓

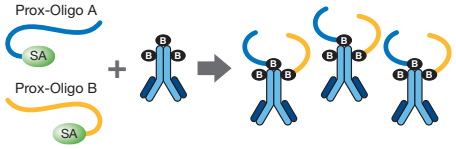
Perform the Forced Proximity Probe Test (page 15)

Dilute the biotinylated antibody (page 15)

↓

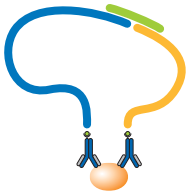
Prepare the Forced Proximity Probe and the negative control (page 16)

↓



Perform the ligation and protease reactions (page 17)

↓

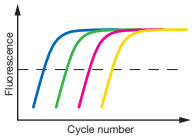


Perform the real-time PCR (page 21)

↓

Analyze the real-time data (page 24)

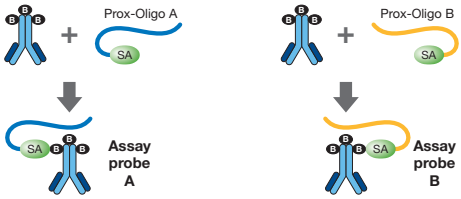
↓



Prepare the Assay Probes (page 27)

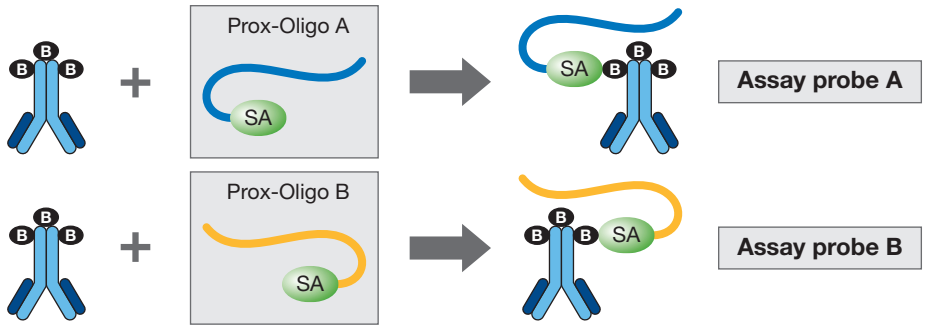
↓ Prepare Assay Probe A

↓ Prepare Assay Probe B



Select and prepare biotinylated antibodies

Each of the two Assay Probes is prepared by combining biotinylated antibodies with either the 5′ or 3′ Prox-Oligo (streptavidin-linked oligos).



IMPORTANT! You must use biotinylated antibodies to prepare the Assay Probes. If the antibody you select is not biotinylated, you must label the non-biotinylated antibody with biotin before continuing with this protocol. See Appendix B, "Select and Prepare Non-Biotinylated Antibodies" on page 39.

Guidelines for selecting biotinylated antibodies

To increase the likelihood of successfully obtaining a working proximity probe set, Applied Biosystems strongly recommends that the biotinylated antibody you select:

- Is a polyclonal antibody or a qualified ELISA antibody pair. If you use a polyclonal antibody, it must be:
  - Antigen-purified
  - Raised against full-length or near full-length antigen
- Does not contain any free biotin. Perform the Forced-Proximity Probe Test (on page 15) to confirm the absence of free biotin.
- Has a suitable positive control; for example, purified antigen and/or known positive sample. (Recommended for assay characterization.)

If you are using lyophilized biotinylated antibodies, see "Resuspend lyophilized biotinylated antibodies" on page 14.

If the antibody you select is not biotinylated, see Appendix B, "Select and Prepare Non-Biotinylated Antibodies" on page 39.

IMPORTANT! Due to the inherent nature of antibodies, Applied Biosystems cannot guarantee that your antibody or antibody pair will result in a working proximity probe set. See Appendix A on page 31 for a list of commercially available antibodies that have been demonstrated to be either successful or unsuccessful in TaqMan® Protein Assays when tested with a suitable positive control.

Resuspend lyophilized biotinylated antibodies

If you are using lyophilized biotinylated antibodies, follow the resuspension procedures below.

1. Before removing the cap, bring the vial of lyophilized biotinylated antibody to room temperature. Keep the vial at room temperature for step 2 through step 3.

2. Carefully add Antibody Dilution Buffer to resuspend the lyophilized antibody. Be sure that any lyophilized material on the cap and sides of the vial is also resuspended. Place the cap back on the vial.

   Note: The volume of Antibody Dilution Buffer to use depends on the quantity of lyophilized biotinylated antibody in the vial. Applied Biosystems recommends that you use enough Antibody Dilution Buffer to bring the final concentration of antibody stock solution to 0.5 mg/mL.

3. Finger-tap or invert the vial 5 to 10 times, repeating occasionally over a period of 5 minutes.

   IMPORTANT! Do not vortex the vial after resuspension.

After resuspending the lyophilized biotinylated antibody:

- (Recommended) Place the biotinylated antibody stock solution on ice, then proceed *immediately* to "Perform the Forced Proximity Probe Test" on page 15.

  OR

- Perform the Forced Proximity Probe Test within 3 days. Store the biotinylated antibody stock solution at 2 to 8°C until you perform the test.

  Note: After the biotinylated antibody has passed the Forced Proximity Probe Test, you can aliquot the stock solution for long-term storage at –80°C. See "Storing the biotinylated antibody after the Forced Proximity Probe Test" on page 26.

Perform the Forced Proximity Probe Test

Perform the Forced Proximity Probe Test to determine whether or not the biotinylated antibody is suitable for making proximity probes.

IMPORTANT! The Forced Proximity Probe Test determines whether or not the biotinylated antibody can bind to the Prox-Oligos. The test cannot determine whether or not the antibody is suitable for use in TaqMan® Protein Assays experiments.

Dilute the biotinylated antibody

Dilute an aliquot of biotinylated antibody stock solution to 200 nM (30 µg/mL):

1. If you are using frozen biotinylated antibody:
   a. Remove an aliquot from storage and immediately place it on ice to thaw.
   b. After the antibody has thawed, gently mix (but do not vortex).
   c. Spin the tube briefly (~5 seconds) to bring the liquid to the tube bottom.
2. Working on ice, combine the components listed below. The volumes listed in the table are provided as a guide.

Component	Volume (µL)
	EXAMPLE For antibody stock solution at 0.5 mg/mL (3.3 µM)
Antibody Dilution Buffer	47
Biotinylated antibody stock solution	3
Total volume of 200 nM biotinylated antibody	**50**

3. Briefly centrifuge to spin the liquid to the tube bottom.
4. Place the 200 nM biotinylated antibody on ice.

After diluting the biotinylated antibody:

- Proceed to "Prepare the Forced Proximity Probe and the negative control" on page 16. You need 2 µL of the 200 nM biotinylated antibody to prepare the Forced Proximity Probe.

  AND
- Store the rest of the 200 nM biotinylated antibody at 4°C for up to 48 hours. After the biotinylated antibody has passed the Forced Proximity Probe Test, you can use the 200 nM biotinylated antibody to prepare the Assay Probes (page 27).

Prepare the Forced Proximity Probe and the negative control

1. Prepare the Prox-Oligo mix:
   a. Working on ice, combine the components listed below.

Component	Volume (µL)
200 nM 3′ Prox-Oligo	5
200 nM 5′ Prox-Oligo	5
Total volume of 200 nM Prox-Oligo mix	**10**

   b. Mix gently, then briefly centrifuge to spin the liquid to the tube bottom.
   c. Place the tube on ice.
2. Label two tubes: **Forced Proximity Probe** and **Negative Control**.
3. Working on ice, combine the components listed below in the appropriate tube.

Component	Volume (µL)	
	Forced Proximity Probe tube	**Negative Control tube**
200 nM Prox-Oligo mix (from step 1 above)	2	2
200 nM Biotinylated antibody (from page 15)	2	--
Antibody Dilution Buffer	--	2
Total volume	**4**	**4**

4. Mix gently, then briefly centrifuge to spin the liquid to the tube bottoms.
5. Incubate the tubes at room temperature for 60 minutes.
6. Add 396 µL of Assay Probe Dilution Buffer to each tube. The total volume in each tube should be 400 µL.
7. Incubate the tubes at room temperature for 30 minutes.
8. Place both tubes on ice.

After preparing the Forced Proximity Probe and the negative control, proceed to "Perform the ligation and protease reactions" on page 17.

Perform the ligation and protease reactions

Guidelines for the ligation and protease reactions

- Follow the guidelines for transferring reagents, as described in "Pipetting guidelines" on page 53.
- Inspect the pipette tips to ensure consistent reagent aspiration and delivery. Volume variation generates significant variations in the threshold cycle (C_T) values calculated by the real-time PCR system.
- Use reservoirs and multichannel pipettes to aliquot the ligation and protease reagents.

IMPORTANT! Keep all reagents on ice when not in use. Do not allow the tubes to warm to room temperature.

IMPORTANT! Keep the reaction plates on ice during reagent transfers.

Perform the ligation reaction

1. Thaw or place the following components on ice:
   - DNA Ligase, 500×
   - Ligase Dilution Buffer, 1×
   - Ligation Reaction Buffer, 20×
   - 1× PBS, pH 7.4 (for protease reactions)
   - Protease, 100×
2. Dilute the DNA Ligase:

   IMPORTANT! Prepare fresh diluted ligase for each experiment.

   a. Combine the components listed below.

Component	Volume (µL)
DNA Ligase, 500×	2
Ligase Dilution Buffer, 1×	198
Total volume of diluted DNA Ligase	**200**

   b. Mix gently.

   c. Place the diluted DNA Ligase on ice.

3. Prepare the ligation solution:
   a. Combine the components listed below.

Component	Volume (µL)
Ligation Reaction Buffer, 20×	50
Nuclease-free water	908
Diluted DNA Ligase (from step 2 above)	2
Total volume of ligation solution	**960**

   b. Invert the tube to mix the components.
   c. Place the ligation solution on ice.

4. Add the ligation solution to a 96-well reaction plate:
   a. Place the reaction plate on ice.
   b. To 8 wells of the reaction plate, add the components as indicated in the table below.

Component	Volume per well (µL)							
	Well 1	**Well 2**	**Well 3**	**Well 4**	**Well 5**	**Well 6**	**Well 7**	**Well 8**
Forced Proximity Probe (from page 16)	4	4	4	4	NA			
Negative Control (from page 16)	NA				4	4	4	4
Total volume per well (µL)	**4**	**4**	**4**	**4**	**4**	**4**	**4**	**4**

   c. Add 96 µL of the ligation solution to each of the 8 reaction wells. Pipet up and down once to mix.

5. Seal the ligation reaction plate with a MicroAmp® Clear Adhesive Film.

6. Briefly centrifuge the sealed reaction plate.

7. Incubate the sealed reaction plate using the thermal-cycling conditions listed in the table below. Be sure to:
   - Use a thermal cycler with a heated cover.
   - Place a MicroAmp® Optical Film Compression Pad on top of the sealed reaction plate to form a tight seal during incubation.

IMPORTANT! To prevent condensation and evaporation, you must use a compression pad and a heated cover when using the thermal cycler. Failure to do so will result in highly variable data.

Step	Cycle number	Temperature (°C)	Time	Reaction volume
Ligation	1	37	10 minutes	Default
Cooling	1	4	Up to 10 minutes	Default

After performing the ligation reaction, proceed to "Perform the protease reaction" on page 20.

Note: You can omit the protease step if you proceed *immediately* to the real-time PCR step on page 21. Otherwise, continue with the protease reaction page 20.

Perform the protease reaction

1. Dilute the protease:
   a. Briefly vortex the protease to mix the solution.
   b. Combine the components listed below.

Component	Volume (µL)
Protease, 100×	1
1× PBS, pH 7.4	99
Total volume of diluted protease solution	**100**

   c. Mix gently, then briefly centrifuge to spin the liquid to the tube bottom.
   d. Place the diluted protease solution on ice.

2. Add the diluted protease solution to the ligation reaction plate to terminate the ligation reaction:
   a. Remove the ligation reaction plate from the thermal cycler.
   b. Remove the MicroAmp® Clear Adhesive Film from the ligation reaction plate, then place the plate on ice.
   c. Add 2 µL of the diluted protease to each of the 8 reaction wells of the ligation reaction plate.

      Note: No mixing is required. The protease will diffuse throughout the samples during the 10-minute incubation.

3. Using a new MicroAmp Clear Adhesive Film, reseal the reaction plate.

4. Incubate the sealed reaction plate using the thermal-cycling conditions provided in the table below. Be sure to:
   - Use a thermal cycler with a heated cover.
   - Place a MicroAmp® Optical Film Compression Pad on top of the sealed reaction plate to form a tight seal during incubation.

IMPORTANT! To prevent condensation and evaporation, you must use a compression pad and a heated cover when using the thermal cycler. Failure to do so will result in highly variable data.

Step	Cycle number	Temperature (°C)	Time	Reaction volume
Terminate ligation	1	37	10 minutes	Default
Inactivate protease	1	95	5 minutes	Default
HOLD	1	4	Hold	Default

5. Remove the reaction plate from the thermal cycler and place it on ice.

After performing the protease reaction:

- Proceed to "Perform the real-time PCR" below.

  OR

- Store the protease-treated ligation products at 4°C for up to 3 days, or at –20°C for up to 2 weeks.

Perform the real-time PCR

Guidelines for performing the real-time PCR

- Follow the guidelines for transferring reagents, as described in "Pipetting guidelines" on page 53.
- Power on your real-time PCR instrument at least 30 minutes before loading the PCR reaction plate.
- (For the 7900HT/7900HT Fast systems) Be sure that the plate-loading door is closed until just before you load the PCR reaction plate.

IMPORTANT! Keep all reagents on ice when not in use. Do not allow the tubes to warm to room temperature.

IMPORTANT! Keep the reaction plates on ice during reagent transfers.

Prepare the PCR mix

1. Thaw the Universal PCR Assay on ice.
2. Combine the components listed below.

Component	Volume (µL)
Fast Master Mix, 2X	100
Universal PCR Assay, 20×	10
Total volume of PCR mix	**110**

3. Mix gently, then briefly centrifuge to spin the liquid to the tube bottom.
4. Place the PCR mix on ice.

Prepare the PCR plate

1. Place a PCR plate on ice.
2. Add 11 µL of the PCR mix to each of 8 wells of the PCR plate.
3. Add the protease-treated ligation product to the PCR plate:
   a. Remove the MicroAmp® Clear Adhesive Film from the protease reaction plate, then place the plate on ice.
   b. Transfer 9 µL of the protease-treated ligation product from each of the 8 reaction wells of the protease reaction plate to each of the 8 reaction wells of the PCR plate. When transferring, pipet up and down once to mix.
4. Seal the PCR plate with a MicroAmp® Optical Adhesive Film (do not use a standard adhesive cover).

5. Briefly centrifuge the PCR plate to combine the solutions and remove bubbles.

   If you perform the run on a 7900HT/7900HT Fast system with a 96-Well Block Module and an automation accessory, place a MicroAmp® Snap-On Optical Film Compression Pad on top of the plate.

IMPORTANT! After preparing the PCR plate, proceed *immediately* to "Run the PCR plate" below.

Run the PCR plate

1. In your real-time PCR system software, create a plate document/experiment for the run. Use the setup information provided in the table below.

   Note: Table 1 on page 23 provides detailed thermal-cycling conditions for each run type listed below.

System	StepOnePlus™	7500 Fast		7900HT Fast	7900HT
Software	StepOne™ Software v1.0 or later	SDS Software v1.4 or later	7500 Software v2.0 or later	SDS Software v2.1 or later	SDS Software v2.0 or later
Template	cDNA	—	cDNA	—	—
Run type	Fast				Standard
Reaction plate	Fast 96-well			Fast 96-well, Standard 96-well, or 384-well	Standard 96-well or 384-well
Sample volume	20 µL				20 µL
Detectors/ targets	Reporter: FAM™ dye Quencher: Non-fluorescent				Reporter: FAM™ dye Quencher: Non-fluorescent
Ramp speed/ mode	Fast			Fast or Standard	Standard
Experiment type	Select an experiment type that will generate C_T values, such as Absolute Quantitation or Standard Curve.				
Tasks and quantities	You do not need to assign tasks or quantities.				
Analysis settings	Threshold cycle (C_T): 0.2 Baseline: Automatic				

2. (For the 7900HT/7900HT Fast systems) Be sure that the plate-loading door is closed until just before you load the PCR reaction plate.

3. Load the PCR plate into your real-time PCR instrument.

4. Start the run.

Table 1 Thermal-cycling conditions

Run type	Reaction plate	Stage	Temperature (°C)	Time (enzyme activation, denaturation, annealing/extension)
StepOnePlus™ system				
Fast	Fast 96-well	Hold	95	20 seconds
		Cycle	95	1 second
		(40 cycles)	60	20 seconds
7500 Fast system				
Fast	Fast 96-well	Hold	95	20 seconds
		Cycle	95	3 seconds
		(40 cycles)	60	30 seconds
7900HT Fast system				
Fast	Fast 96-well	Hold	95	20 seconds
		Cycle	95	1 second
		(40 cycles)	60	20 seconds
7900HT system				
Standard	Standard 96-well or 384 well	Hold	95	2 minutes
		Cycle	95	15 seconds
		(40 cycles)	60	1 minute

Analyze the real-time data

Analyze the data from the Forced Proximity Probe Test using the real-time PCR system software and a spreadsheet program.

Using the real-time PCR system software

For all real-time PCR systems:

1. View the amplification plots for the entire reaction plate.
2. Analyze the plate run using a threshold cycle (C_T) setting of 0.2 and automatic baseline.

Using a spreadsheet program

1. Export the results from the instrument software to a spreadsheet program (such as Microsoft® Excel® software).
2. Calculate the average C_T values for each biotinylated antibody and negative control.
3. Calculate the ΔC_T values for each biotinylated antibody:
   $AvgC_T$(negative control) – $AvgC_T$(Forced Proximity Probe)

Expected results

Use the table below as a guide to determine if the biotinylated antibody passed the Forced Proximity Probe Test. From day-to-day, the absolute C_T values for both the Forced Proximity Probe and the negative control may shift up or down. However, the ΔC_T value [AvgC_T (negative control) – AvgC_T (Forced Proximity Probe)] should remain constant.

Note: See Appendix C on page 47 for example data.

ΔC_T value	Result	Comment	
≥8.5	Pass	The biotinylated antibody is suitable for use in TaqMan® Protein Assays experiments.	
<8.5	Fail	**Possible causes**	• There is excess free biotin in the preparation (for example, if the biotin-antibody conjugate has not undergone extensive dialysis). The 5′ and 3′ Prox-Oligos will bind to the free biotin instead of the biotinylated antibodies, leading to a mixed population of oligo-labeled and unlabeled antibodies. • The antibody is not biotinylated. • The antibody concentration is not correct.
		Recommended actions	Be sure that the concentration of the biotinylated antibody is correct. If needed, use a total protein quantitation assay (such as the Micro BCA™ Protein Assay Kit) to determine the correct concentration. **If the concentration is not correct,** repeat the Forced Proximity Probe Test with the correct amount of biotinylated antibody. **If the concentration is correct:** 1. Redialyze the biotinylated antibody for an additional 1 to 2 buffer changes. 2. Perform the Forced Proximity Probe Test again.
			If the biotinylated antibody fails the Forced Proximity Probe Test a second time, the antibody may not be biotinylated. Prepare or obtain more biotinylated antibody, then perform the Forced Proximity Probe Test again.

Storing the biotinylated antibody after the Forced Proximity Probe Test

After the biotinylated antibody has passed the Forced Proximity Probe Test:

- Use the biotinylated antibody to prepare the Assay Probes; proceed to "Prepare the Assay Probes" on page 27.

  OR

- Dispense and store the biotinylated antibody for long-term storage; see "Long-term storage" below.

Long-term storage

Applied Biosystems recommends long-term storage of the biotinylated antibody stock solution only after you obtain satisfactory results with the Forced Proximity Probe Test.

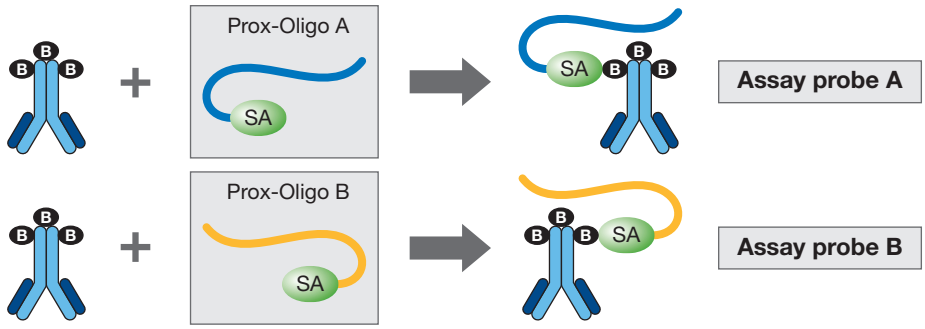
1. (This step is required only if the biotinylated antibody does not contain carrier protein.) Add carrier protein to 0.1% (1 mg/mL). For example, add 2 µL of 10% BSA to 200 µL of biotinylated antibody.

2. Aliquot small volumes (5 to 10 µL) to the bottom of tubes with screw caps.
   - Do not use caps with O-rings. Applied Biosystems recommends NALGENE 2-mL or 0.5-mL tubes with caps.
   - Do not centrifuge the tubes.

3. Store the biotinylated antibody stock solution at –80°C.

Prepare the Assay Probes

Each of the two Assay Probes is prepared by combining biotinylated antibodies with either the 5′ or 3′ Prox-Oligo (streptavidin-linked oligos).



Prepare Assay Probes A and B for each biotinylated polyclonal antibody or qualified ELISA antibody pair that has passed the Forced Proximity Probe Test.

Guidelines

- Before you begin, read through the entire procedure.
- During the procedure, keep the materials on ice as much as possible.
- To save time, you can assemble Assay Probe A and Assay Probe B at the same time.

IMPORTANT! If you assemble Assay Probes A and B at the same time, be extremely careful not to cross-contaminate the Prox-Oligos. Change the pipette tips between each addition.

Prepare Assay Probe A

1. Briefly centrifuge the 200 nM biotinylated antibody (from page 15) to spin the liquid to the tube bottom, then place the tube on ice.
2. Briefly centrifuge the 3′ Prox-Oligo to spin the liquid to the tube bottom, then place the tube on ice.
3. Working on ice, combine the components listed below.

Component	Volume (µL)
200 nM Biotinylated antibody (from page 15)	5
200 nM 3′ Prox-Oligo	5
Total volume	**10**

4. Mix gently, then briefly centrifuge to spin the liquid to the tube bottom.
5. Incubate the tube at room temperature for 60 minutes.
6. Allow the Assay Probe Storage Buffer to come to room temperature.
7. Add 90 µL of Assay Probe Storage Buffer to the tube, then mix gently. The total volume should be 100 µL.
8. Briefly centrifuge to spin the liquid to the tube bottom.
9. Incubate the tube at room temperature for 20 minutes.
10. Store Assay Probe A at –20°C for up to 6 months.

Prepare Assay Probe B

1. Briefly centrifuge the 200 nM biotinylated antibody (from page 15) to spin the liquid to the tube bottom, then place the tube on ice.
2. Briefly centrifuge the 5′ Prox-Oligo to spin the liquid to the tube bottom, then place the tube on ice.
3. Working on ice, combine the components listed below.

Component	Volume (µL)
200 nM Biotinylated antibody	5
200 nM 5′ Prox-Oligo	5
Total	**10**

4. Mix gently, then briefly centrifuge to spin the liquid to the tube bottom.
5. Incubate the tube at room temperature for 60 minutes.
6. Allow the Assay Probe Storage Buffer to come to room temperature.
7. Add 90 µL of Assay Probe Storage Buffer to the tube, then mix gently. The total volume should be 100 µL.
8. Briefly centrifuge to spin the liquid to the tube bottom.
9. Incubate the tube at room temperature for 20 minutes.
10. Store Assay Probe B at –20°C for up to 6 months.

Screened Antibodies

This appendix covers:

About the screened antibodies

This appendix provides a list of commercially available antibodies that have been demonstrated to be either successful or unsuccessful in TaqMan® Protein Assays when tested with a suitable positive control.

The positive control is either recombinant protein in buffer or lysate from cells or tissues that are known or expected to express the target. The passing criteria for:

- Recombinant protein is $\Delta C_T > 5$
- Cell or tissue lysates is $\Delta C_T > 3$

Where: $\Delta C_T = C_T(\text{NPC}) - C_T(\text{test sample})$

The ΔC_T values will generally be lower for cell or tissue lysate samples than for recombinant protein for a given target because of interference of the biological matrix.

IMPORTANT! Due to the inherent nature of antibodies, Applied Biosystems cannot guarantee that your antibody or antibody pair will result in a working proximity probe set.

Where to go for updates

The antibody list in this appendix is periodically updated on the Applied Biosystems Web site. To view the most current list, go to:

www.appliedbiosystems.com/taqman4protein

Screened antibodies

The table below lists 89 antibodies that:

- Meet the antibody selection criteria described in "Guidelines for selecting biotinylated antibodies" on page 13.
- Have been tried in a TaqMan® Protein Assay with positive controls.

The overall success rate with these antibodies is 74%.

Target	Species	Antibody source and description						Immunoassay validation (from vendor)				TaqMan® Protein Assay results§		
		Source	Part no.	Type†	Host	Biotin-labeled‡	Immunogen	WB	ELISA	IHC	Flow cytometry	Recombinant protein (ΔC_T >5)	Biological sample (ΔC_T >3)††	OVERALL PASS/FAIL
ADAM9	Human	R&D Systems	BAF939	pAb	Goat	Yes	Partial	Yes				+	+	+
ADAM9	Mouse	R&D Systems	BAF949	pAb	Goat	Yes	Partial	Yes			Yes	NA	+	+
ALCAM/ CD166	Human	R&D Systems	BAF656	pAb	Goat	Yes	Partial	Yes	Yes			+	+	+
beta catenin	Human	R&D Systems	AF1329	pAb	Goat	No	Full length	Yes		Yes	Yes	–	–	–
BMI-1	Human	R&D Systems	AF3334	pAb	Goat	No	Partial	Yes	Yes		Yes	–	–	–
BRACH-YURY	Human	R&D Systems	BAF2085	pAb	Goat	Yes	Full length	Yes				–	–	–
CA2	Human	R&D Systems	BAF2184	pAb	Goat	Yes	Partial	Yes				+	NA	+
Calci-neurin B	Human/ mouse	R&D Systems	AF1348	pAb	Rabbit	No	Full length	Yes				+	–	–
CASP8	Human	R&D Systems	AF705	pAb	Goat	No	Full length	Yes				+	NA	+
CASP8	Human	R&D Systems	AF1650	pAb	Rabbit	No	Full length	Yes				–	NA	–
CASPASE 3 (ACTIVE) D175	Human	Invitrogen	700182	mAb	Rabbit	No	Peptide		Yes			+	–	+ (1)
CASPASE 3 (total)	Human	R&D Systems	AF605NA	pAb	Goat	No	Full length	Yes				+	+	+
Cathepsin B	Human	R&D Systems	BAF953	pAb	Goat	Yes	Full length	Yes		Yes		+	+	+

Target	Species	Antibody source and description						Immunoassay validation (from vendor)				TaqMan® Protein Assay results§		
		Source	Part no.	Type†	Host	Biotin-labeled‡	Immunogen	WB	ELISA	IHC	Flow cytometry	Recombinant protein (ΔC_T >5)	Biological sample (ΔC_T >3)††	OVERALL PASS/FAIL
Cathepsin B	Mouse	R&D Systems	BAF965	pAb	Goat	Yes	Partial	Yes		Yes		+	+	+
Cathepsin D	Human	R&D Systems	BAF1014	pAb	Goat	Yes	Full length	Yes		Yes		+	+	+
Cathepsin D	Mouse	R&D Systems	BAF1029	pAb	Goat	Yes	Full length	Yes		Yes		+	+	+
CCL5	Human	R&D Systems	BAF278	pAb	Goat	Yes	Full length	Yes	Yes			+	NA	+
CD105/ ENDO-GLIN	Human	R&D Systems	BAF1097	pAb	Goat	Yes	Partial	Yes	Yes	Yes		+	+	+
CD29 (ITGNB)	Human	R&D Systems	BAF1778	pAb	Goat	Yes	Partial	Yes			Yes	–	+	+
CD30/ TNFSF8	Human	R&D Systems	BAF1028	pAb	Goat	Yes	Partial	Yes				+	+	+
CD48	Human	R&D Systems	AF3644	pAb	Goat	No	Full length	Yes	Yes		Yes	+		+
CKIT (CD117)	Human	R&D Systems	BAF332	pAb	Goat	Yes	Partial	Yes	Yes	Yes		+	+	+
ckit (CD117)	Mouse	R&D Systems	BAF1356	pAb	Goat	Yes	Partial	Yes		Yes	Yes	NA	–	–
c-Myc	Human	R&D Systems	AF3696	pAb	Goat	No	Partial	Yes	Yes	Yes		–	–	–
CRIPTO-1	Human	R&D Systems	BAF145	pAb	Goat	Yes	Full length	Yes				–	NA	–
CRIPTO-1	Human	R&D Systems	BAM2773	mAb (d)	Mouse	Yes	Full length		Yes			–	NA	–
CRIPTO-1	Human	R&D Systems	MAB2772	mAb (c)	Mouse		Full length		Yes			–	NA	–
CSTB	Human	R&D Systems	BAF1408	pAb	Goat	Yes	Full length	Yes				+	+	+
DECORIN	Human	R&D Systems	BAF143	pAb	Goat	Yes	Full length	Yes				+	+	+ (2)
DECORIN	Human	R&D Systems	BAM1431	mAb (d)	Mouse	Yes	Full length		Yes			+	+	+ (3)
DECORIN	Human	R&D Systems	MAB1432	mAb (c)	Mouse	No	Full length		Yes			+	+	+
Dppa4	Human	R&D Systems	BAF3674	pAb	Sheep	Yes	Full length	Yes			Yes	NA	+	+

Target	Species	Antibody source and description						Immunoassay validation (from vendor)				TaqMan® Protein Assay results§		
		Source	Part no.	Type†	Host	Biotin-labeled‡	Immunogen	WB	ELISA	IHC	Flow cytometry	Recombinant protein (ΔC_T >5)	Biological sample (ΔC_T >3)††	OVERALL PASS/FAIL
E-Cadherin (CDH1)	Human	R&D Systems	BAF648	pAb	Goat	Yes	Partial	Yes	Yes			+	+	+
EGF	Human	R&D Systems	BAF236	pAb	Goat	Yes	Full length	Yes	Yes			+	NA	+
EGFR	Human	R&D Systems	BAF231	pAb	Goat	Yes	Partial	Yes	Yes		Yes	+	+	+
EPCAM	Human	R&D Systems	BAF960	pAb (d)	Goat	Yes	Partial	Yes	Yes		Yes	NA	+	+ (4)
EPCAM	Human	R&D Systems	MAB9601	mAb	Mouse	No	Partial		Yes		Yes	NA	+	+ (5)
ERK1	Human	R&D Systems	BAF1879	pAb	Goat	Yes	Full length	Yes				–	–	–
FOXA2	Human	R&D Systems	BAF2400	pAb	Goat	Yes	Partial	Yes				NA	–	–
GATA4	Human	R&D Systems	BAF2606	pAb	Goat	Yes	Partial	Yes		Yes		NA	+	+
GFAP (glial fibr. Acid protein)	Human	R&D Systems	BAF2594	pAb	Sheep	Yes	Partial	Yes		Yes		–	–	–
HDAC8	Human/ Mouse	R&D Systems	BAF4359	pAb	Sheep	Yes	Full length	Yes				+	–	+
ICAM-1	Human	R&D Systems	BAF720	pAb	Sheep	Yes	Partial	Yes	Yes			+	+	+
ICAM-1	Mouse	R&D Systems	BAF796	pAb	Goat	Yes	Partial	Yes	Yes	Yes		+	+	+
IFN-GAMMA	Human	R&D Systems	BAF285	pAb	Goat	Yes	Full length	Yes	Yes	Yes		–	NA	–
IGF-II	Human	R&D Systems	BAF292	pAb	Goat	Yes	Full length	Yes				–	NA	–
IL-10	Human	R&D Systems	BAF217	pAb	Goat	Yes	Full length	Yes	Yes			+	NA	+
IL-12	Human	R&D Systems	BAF219	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+
IL-1a	Human	R&D Systems	BAF200	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+
IL1B	Human	R&D Systems	BAF201	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+

Target	Species	Antibody source and description						Immunoassay validation (from vendor)				TaqMan® Protein Assay results§		
		Source	Part no.	Type†	Host	Biotin-labeled‡	Immunogen	WB	ELISA	IHC	Flow cytometry	Recombinant protein (ΔC_T >5)	Biological sample (ΔC_T >3)††	OVERALL PASS/FAIL
IL2	Human	R&D Systems	BAF202	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+
IL4	Human	R&D Systems	BAF204	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+
IL6	Human	R&D Systems	BAF206	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+
IL-7	Human	R&D Systems	BAF207	pAb	Goat	Yes	Full length	Yes	Yes			+	NA	+
IL8	Human	R&D Systems	BAF208	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+
KLF-4	Human	R&D Systems	AF3640	pAb	Goat	No	Full length	Yes	Yes	Yes		–	+	+
LIN28	Human/ Mouse	R&D Systems	BAF3757	pAb	Goat	Yes	Full length	Yes				+	+	+
MCP1/ CCL2	Human	R&D Systems	BAF279	pAb	Goat	Yes	Full length	Yes	Yes			+	NA	+
MMP-9	Human	R&D Systems	BAF911	pAb	Goat	Yes	Full length	Yes	Yes			+	NA	+
NANOG	Human	R&D Systems	BAF197	pAb	Goat	Yes	Partial	Yes		Yes		+	+	+
NANOG	Mouse	R&D Systems	BAF2729	pAb	Goat	Yes	Partial	Yes				NA	–	–
NCAM-1	Human	R&D Systems	BAF2408	pAb	Goat	Yes	Full length	Yes	Yes			+	+	+
Nestin	Human	GeneTex	31789	pAb	Rabbit	Yes	Partial					NA	–	–
NGF R	Human	R&D Systems	BAF367	pAb	Goat	Yes	Partial	Yes				+	–	+
NOGO R	Human	R&D Systems	BAF1208	pAb	Goat	Yes	Partial	Yes				+	–	+
Notch-1	Human	R&D Systems	BAF3647	pAb	Sheep	Yes	Partial	Yes				–	–	–
OCT3/4	Human	R&D Systems	BAF1759	pAb	Goat	Yes	Full length	Yes		Yes		NA	+	+
OPG/ TNFRSF 11B	Human	R&D Systems	BAF805	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+

Target	Species	Antibody source and description						Immunoassay validation (from vendor)				TaqMan® Protein Assay results§		
		Source	Part no.	Type†	Host	Biotin-labeled‡	Immunogen	WB	ELISA	IHC	Flow cytometry	Recombinant protein (ΔC_T >5)	Biological sample (ΔC_T >3)††	OVERALL PASS/FAIL
p21	Human	R&D Systems	BAF1047	pAb	Goat	Yes	Full length	Yes		Yes		–	NA	–
p53	Human/ Mouse	R&D Systems	BAF1355	pAb	Goat	Yes	Full length	Yes				+	+	+
PD-1	Human	R&D Systems	BAF1086	pAb	Goat	Yes	Partial	Yes	Yes		Yes	+	NA	+
PDGF-BB	Human	PeproTech	500-P47Bt	pAb	Rabbit	Yes	Full length	Yes	Yes			+	NA	+
SCF	Human	R&D Systems	BAF255	pAb	Goat	Yes	Full length	Yes	Yes			+	–	+
SMAC/ DIABLO	Human	R&D Systems	BAF789	pAb	Goat	Yes	Full length	Yes				+	NA	+
SOX17	Human	R&D Systems	BAF1924	pAb	Goat	Yes	Partial	Yes		Yes		NA	–	–
SOX2	Human/ Mouse	R&D Systems	BAF2018	pAb	Goat	Yes	Partial	Yes		Yes		NA	+	+
Survivin	Human	R&D Systems	AF6471	pAb	Goat	No	Full length	Yes	Yes		Yes	NA	+	+
Survivin	Human	R&D Systems	AF866	pAb	Rabbit	No	Full length	Yes		Yes		NA	–	–
TGFB1	Human	Invitrogen	CHC1763	PAIR	NA	Yes/No	NA		Yes			+	NA	+
TGF-BETA	Human	R&D Systems	BAF240	pAb	Chicken	Yes	Full length	Yes	Yes			–	NA	–
TNF RI	Human	Invitrogen	CHC1683	PAIR	NA	Yes/No	NA		Yes			+	NA	+
TNF RI	Human	R&D Systems	BAF225	pAb	Goat	Yes	Full length	Yes	Yes			+	+	+
TNFA	Human	Invitrogen	CHC1753	PAIR	NA	Yes/No	NA		Yes			+	NA	+
TNF-ALPHA	Human	R&D Systems	BAF210	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	NA	+
TROY	Human	R&D Systems	BAF1548	pAb	Goat	Yes	Partial	Yes				+	–	+
VEGF	Human	R&D Systems	BAF293	pAb	Goat	Yes	Full length	Yes	Yes			+	NA	+
Vimentin	Human	R&D Systems	AF2105	pAb	Goat	No	Full length	Yes	Yes	Yes		–	–	–

Target	Species	Antibody source and description						Immunoassay validation (from vendor)				TaqMan® Protein Assay results§		
		Source	Part no.	Type†	Host	Biotin-labeled‡	Immunogen	WB	ELISA	IHC	Flow cytometry	Recombinant protein (ΔC_T >5)	Biological sample (ΔC_T >3)††	OVERALL PASS/FAIL
GFP	Tag	Abcam	AB6658	pAb	Goat	Yes	Full length	Yes	Yes	Yes		+	+	+
		Novus Biologicals	NB100-1365		Rabbit									
GST	Tag	GenScript	A00202	pAb	Rabbit	Yes	Full length	Yes	Yes			+	+	+

† For the antibody type: c = capture antibody and d = detection antibody (when used in ELISA).
‡ Non-biotinylated antibodies were labeled using the procedure outlined in this protocol.
§ The passing criteria is based on ΔC_T, where $\Delta C_T = C_T(NPC) - C_T(test\ sample)$. The test sample is either lysate from 500 cells or 1000 ng of tissue (per well).
†† Using cells or tissues known or expected to express the target.
(1) When paired with AF605NA.
(2) When paired with BAM1431.
(3) When paired with BAF143.
(4) When paired with MAB9601.
(5) When paired with BAF960.

Select and Prepare Non-Biotinylated Antibodies

IMPORTANT! If the antibody you select is not biotinylated, you must prepare the non-biotinylated antibody as described in this appendix before performing the Forced Proximity Probe Test.

This appendix covers:

User-supplied materials

The materials listed in this section are required to perform the procedures in this appendix, but are not included in the TaqMan® Protein Assays kits. Unless otherwise indicated, all items are available from major laboratory suppliers (MLS).

Note: For the SDS (Safety Data Sheet) of any chemical not distributed by Applied Biosystems, contact the chemical manufacturer. Before handling any chemicals, refer to the SDS provided by the manufacturer, and observe all relevant precautions.

Antibodies without carrier protein

If you are using antibody that is free of carrier protein and in an amine-free buffer (for example, no BSA, gelatin, glycine, Tris, or azide >0.1%), use the materials listed below.

Item	Source
One of the following biotinylation kits:	
• EZ-Link™ Sulfo-NHS-LC-Biotin, No-Weigh™ Format	Pierce (PN 21327)
• Biotin-XX Microscale Protein Labeling Kit	Invitrogen (PN B30010)
1✕ PBS (phosphate-buffered saline), pH 7.4	MLS
(Recommended) Slide-A-Lyzer® Mini Dialysis Unit (MWCO=7000)	Thermo Scientific (PN 69562)
Pipettes and pipette tips	MLS
Microfuge tubes	MLS
Microcentrifuge	MLS
Carrier protein (such as BSA)	MLS
Ultrapure water	MLS
(Recommended) Micro BCA™ Protein Assay Kit	Pierce (PN 23235)

Antibodies with carrier protein

If you are using antibody that contains carrier protein, use the materials listed below.

Item	Source
APEX® Biotin-XX Antibody Labeling Kit	Invitrogen (PN A10495)
Pipettes and pipette tips	MLS
Microfuge tubes	MLS
Microcentrifuge	MLS
Carrier protein (such as BSA)	MLS
Ultrapure water	MLS
(Recommended) Micro BCA™ Protein Assay Kit	Pierce (PN 23235)

Working with non-biotinylated antibodies

Antibodies without carrier protein

Antibodies formulated without carrier protein can be biotinylated with the Biotin-XX Microscale Protein Labeling Kit or the EZ-Link™ Sulfo-NHS-LC-Biotin, No-Weigh™ Format. To biotinylate your antibody, follow the guidelines listed below and "Guidelines for labeling non-biotinylated antibodies" on page 43.

- The antibody must be in an amine-free buffer such as 1 × PBS, pH7.4 (no glycine, Tris, or azide >0.1%).
- The antibody should be at ≥0.5 µg/µL.
- Use dialysis to remove excess biotin (see "Perform dialysis to remove free biotin" on page 46), rather than spin/column chromatography.

Note: If you are using lyophilized non-biotinylated antibodies, see "Resuspend lyophilized non-biotinylated antibodies" on page 42.

Antibodies with or without carrier protein

Antibodies formulated with or without carrier protein can be biotinylated with the APEX® Biotin-XX Antibody Labeling Kit. To biotinylate your antibody, follow the guidelines listed below and the protocol provided with the APEX® kit.

- Use 10 to 20 µg of IgG antibody in ≤ 10 µL of neutral pH buffer such as PBS, Tris-buffered saline (TBS), Tris-HCl, HEPES, borate, or equivalent.
- The sample can contain serum or other stabilizing proteins such as BSA or gelatin.
- It may not be necessary to dialyze the antibody after biotinylation with the APEX® kit. Dialyze the biotinylated antibody only if it fails the Forced Proximity Probe Test.

Note: If you are using lyophilized non-biotinylated antibodies, see "Resuspend lyophilized non-biotinylated antibodies" on page 42.

Resuspend lyophilized non-biotinylated antibodies

If you are using lyophilized non-biotinylated antibodies, follow the resuspension procedures below.

IMPORTANT! When resuspending lyophilized non-biotinylated antibodies, use 1X PBS, pH 7.4 (user-supplied). Do not use the Antibody Dilution Buffer for resuspending non-biotinylated antibodies.

---

1. Before removing the cap, bring the vial of lyophilized non-biotinylated antibody to room temperature. Keep the vial at room temperature for step 2 through step 3.

2. Carefully add 1X PBS, pH 7.4 (user-supplied), to resuspend the lyophilized antibody. Be sure that any lyophilized material on the cap and sides of the vial is also resuspended. Place the cap back on the vial.

   Note: The volume of 1X PBS, pH 7.4, to use depends on the quantity of lyophilized non-biotinylated antibody in the vial. Applied Biosystems recommends that you use enough 1X PBS to bring the final concentration of resuspended antibody to:
   - 0.5 mg/mL if you are using the Biotin-XX Microscale Protein Labeling Kit or the EZ-Link™ Sulfo-NHS-LC-Biotin, No-Weigh™ Format
   - 1 to 2 mg/mL if you are using the APEX® Biotin-XX Antibody Labeling Kit

3. Finger-tap or invert the vial 5 to 10 times, repeating occasionally over a period of 5 minutes.

   IMPORTANT! Do not vortex the vial after resuspension.

After resuspending the lyophilized non-biotinylated antibody, proceed *immediately* to "Guidelines for labeling non-biotinylated antibodies" on page 43.

Guidelines for labeling non-biotinylated antibodies

This section provides general guidelines for labeling non-biotinylated antibodies without carrier protein using the Biotin-XX Microscale Protein Labeling Kit or the EZ-Link™ kit. For an example protocol, see page 45.

Note: For labeling non-biotinylated antibodies formulated with carrier protein, use the APEX® Biotin-XX Antibody Labeling Kit and follow the protocol provided with the APEX® kit. It may not be necessary to dialyze the antibody after biotinylation with the APEX® kit. Dialyze the biotinylated antibody only if it fails the Forced Proximity Probe Test.

1. Label the antibody with biotin. Applied Biosystems recommends that you use the Biotin-XX Microscale Protein Labeling Kit or the EZ-Link™ kit. When using either kit:
   - Follow the manufacturer's instructions.
   - The recommended volume for the biotin labeling reaction is 200 µL.
   - Do not use <50 µg of antibody per 200-µL reaction.
   - Use a biotin:antibody molar ratio of 20:1.

2. Remove free biotin using extensive dialysis (column chromatography is not recommended). When performing dialysis, follow these guidelines:
   - (Recommended) Use the Slide-A-Lyzer® Mini Dialysis Unit.
   - Perform dialysis at 4°C in cold 1× PBS, pH 7.4 (user-supplied). Change the buffer at least five times, as follows:

Number of buffer changes	Interval time	Volume (mL) of 1× PBS
Four	2 hours	500
One	Overnight†	1000

† The overnight interval does not need to be the last buffer change.

   - Do not perform dialysis on more than three biotinylated antibody preparations within the same vessel.

3. After dialysis:
   - Measure the recovered volume and recalculate the antibody concentration, as needed.
   - For a more precise determination of protein concentration, perform protein quantitation using the Micro BCA™ Protein Assay Kit.

4. After biotinylation:
   - (Recommended) Place the biotinylated antibody stock solution on ice, then proceed *immediately* to "Perform the Forced Proximity Probe Test" on page 15.

     OR
   - Perform the Forced Proximity Probe Test within 3 days. Store the biotinylated antibody stock solution at 2 to 8°C until you perform the test.

     Note: After the biotinylated antibody has passed the Forced Proximity Probe Test, you can aliquot the stock solution for long-term storage at –80°C. See "Storing the biotinylated antibody after the Forced Proximity Probe Test" on page 26.

Example protocol

Materials used in this example protocol

- EZ-Link™ Sulfo-NHS-LC-Biotin, No-Weigh™ Format
- Slide-A-Lyzer® Mini Dialysis Unit (MWCO=7000)

Guidelines for preparing the biotin reagent

- The recommended biotin:antibody molar ratio is 20:1.
- Resuspend the biotin reagent just before use.

Calculate the amount of biotin to add to your sample

The table below provides an example calculation.

Variable	Calculation	Example
Amount of antibody	–	0.1 mg
Volume of antibody	–	0.2 mL
Concentration of antibody	–	0.5 mg/mL
Molecular weight of antibody	–	150,000 Da
Amount of biotin to add for 20-fold molar excess	((Volume of antibody × Concentration of antibody)/ Molecular weight of antibody) × 20 ((0.2 mL × 0.5 mg/mL)/150,000 g/mol) × 20	1.33×10^{-5} mmoles
Volume of 10-mM biotin reagent to add	(Amount of biotin to add/10 mM) × 1 × 10^6 (1.33×10^{-5} mmoles/10 mM) ×1 × 10^6	1.33 µL

Prepare the biotin labeling reaction

The recommended volume for the biotin labeling reaction is 200 µL.

1. Just before use, resuspend the biotin reagent in 180 µL of ultrapure water (for a final concentration of 10 mM).
2. Add 1.33 µL of 10-mM biotin reagent to 0.2 mL of 0.5 mg/mL antibody.
3. Mix gently, then centrifuge briefly.
4. Incubate at room temperature for 30 to 60 minutes.

Perform dialysis to remove free biotin

1. Carefully transfer the biotinylated antibody solution to the Slide-A-Lyzer Mini Dialysis Unit:
   - Transfer the solution to the chamber bottom.
   - Do not let the pipette tip touch the membrane.
2. Cap the chamber, then place it in a floating holder.
3. Place the holder in a beaker that contains a stir bar.
4. Perform dialysis at 4°C in cold 1× PBS, pH 7.4 (user-supplied). Change the buffer at least five times, as follows:

Number of buffer changes	Interval time	Volume (mL) of 1× PBS
Four	2 hours	500
One	Overnight†	1000

† The overnight interval does not need to be the last buffer change.

5. Carefully transfer the biotinylated antibody preparation to a new microfuge tube.
6. Note any change in volume and recalculate the antibody concentration.

After biotinylation:

- (Recommended) Place the biotinylated antibody stock solution on ice, then proceed *immediately* to "Perform the Forced Proximity Probe Test" on page 15.

  OR
- Perform the Forced Proximity Probe Test within 3 days. Store the biotinylated antibody stock solution at 2 to 8°C until you perform the test.

  Note: After the biotinylated antibody has passed the Forced Proximity Probe Test, you can aliquot the stock solution for long-term storage at –80°C. See "Storing the biotinylated antibody after the Forced Proximity Probe Test" on page 26.

Example Forced Proximity Probe Test and TaqMan® Protein Assay Data

This appendix covers:

Overview

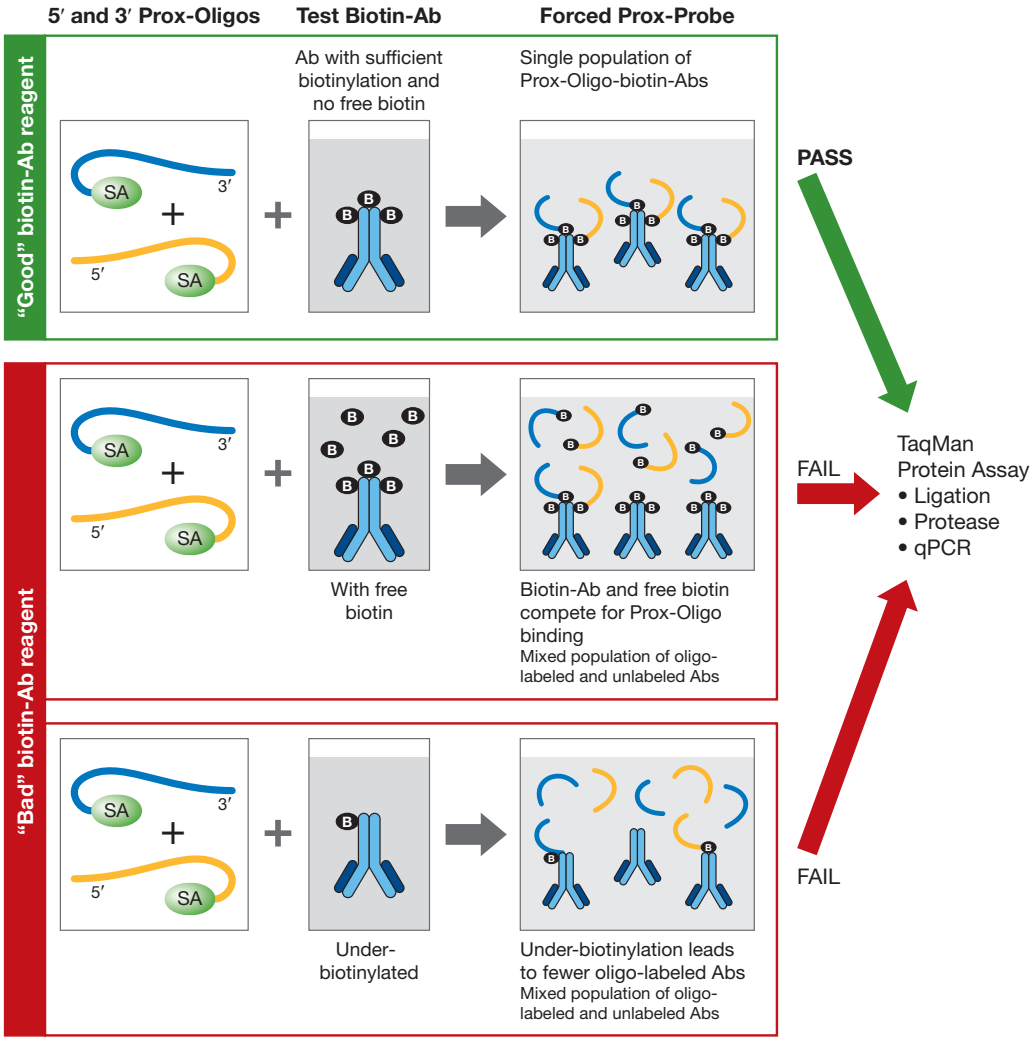
It is very important that you prepare the Assay Probes with antibodies that:

- Do not contain free biotin
- Are sufficiently biotinylated

Using antibodies that contain free biotin or antibodies that are under-biotinylated will result in a mixed population of oligo-labeled and unlabeled antibodies.

About the Forced Proximity Probe Test

The Forced Proximity Probe Test assesses the quality of the biotinylated antibody preparation. The test fails if free biotin is in the preparation or if the antibody is under-biotinylated. See the figure below.



About this example

The example in this appendix illustrates the importance of removing excess biotin from the antibody preparation. In this example, varying amounts of biotin were spiked into solutions of pre-biotinylated CSTB antibody, resulting in biotinylated antibody preparations that contained varying amounts of free biotin. These antibody preparations were then assessed using the Forced Proximity Probe Test and TaqMan® Protein Assays.

Forced Proximity Probe Test data

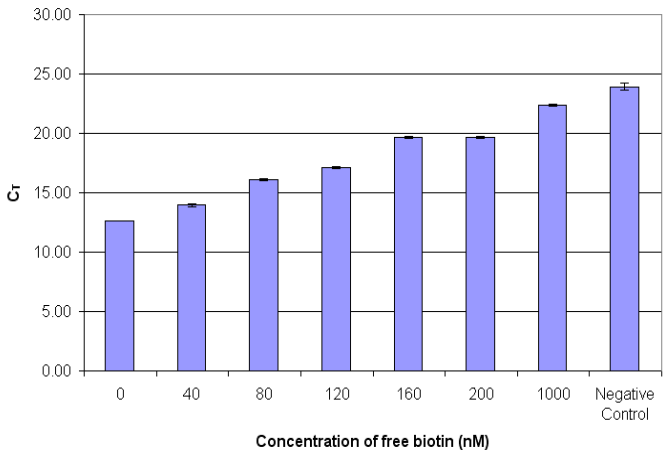
The table below lists Forced Proximity Probe Test data for biotinylated antibody preparations that contained varying amounts of free biotin.

Concentration of free biotin	C_T value	Standard deviation	ΔC_T value†
0 nM	12.66	0.00	11.24
40 nM	13.95	0.12	10.14
80 nM	16.12	0.06	7.81
120 nM	17.10	0.07	7.08
160 nM	19.66	0.08	4.30
200 nM	19.70	0.08	4.41
1000 nM	22.37	0.05	1.01
Negative control	23.94	0.27	NA

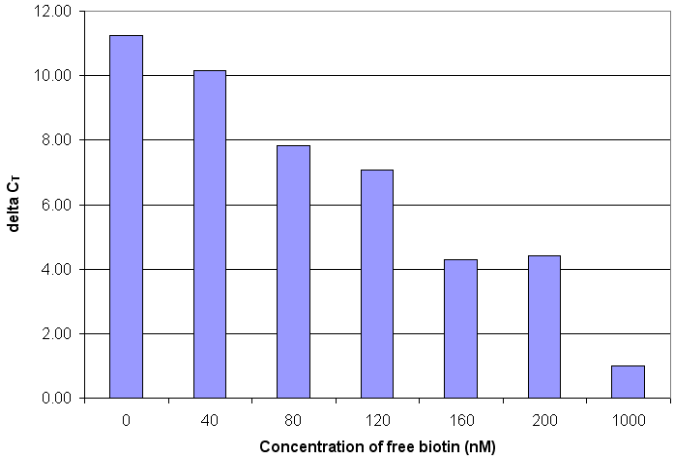
† The ΔC_T value is calculated as follows: C_T(negative control) – C_T(sample).

The negative control does not contain any antibody. The observed C_T value of the negative control represents the background ligation occurring between the 5′ and 3′ Prox-Oligos in the absence of biotinylated antibody. See the figure below.

Note: The C_T value of the Forced Proximity Probe Test negative control is typically lower than the C_T value of a TaqMan® Protein Assay negative control because more ligase is used in the Forced Proximity Probe Test.



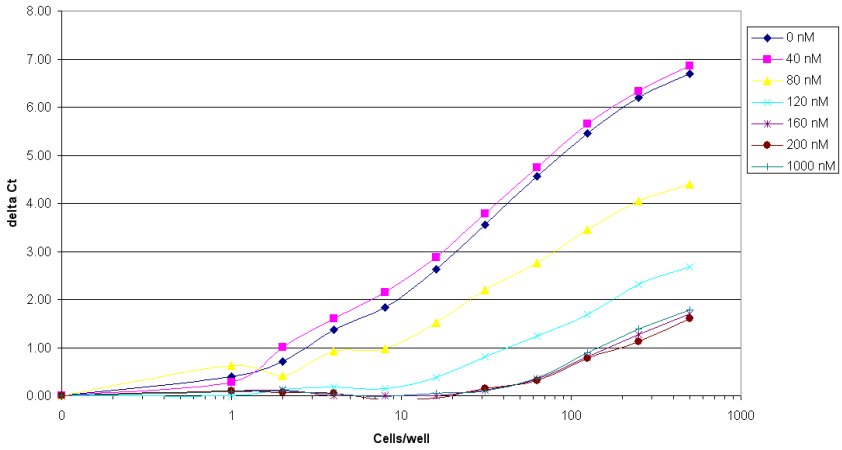
Without any excess biotin, the ΔC_T value for the biotinylated CSTB antibody in the example experiment was 11.24. The ΔC_T value is highly dependant on the biotinylation state of the antibody. With increasing amounts of excess biotin added to the antibody preparation, the ΔC_T values decrease. See the figure below.



TaqMan® Protein Assay data

Applied Biosystems performed TaqMan® Protein Assay experiments with Raji cell lysate and Assay Probes that were prepared with the same biotinylated antibodies used in the Forced Proximity Probe Test. (These antibodies contained varying amounts of free biotin.)

As shown in the figure below, the assay performance noticeably deteriorates when used with Assay Probes prepared with antibodies that contained just 80 nM of free biotin. The ΔC_T value for the Forced Proximity Probe Test of the antibody preparation containing 80 nM of free biotin is 7.81. Consequently, Applied Biosystems recommends preparing Assay Probes with biotinylated antibodies that have Forced Proximity Probe Test ΔC_T values of ≥8.5.



Recommendations

The performance of the TaqMan® Protein Assay is severely compromised when even a small amount of free biotin is present. Applied Biosystems recommends that you:

- Remove free biotin by extensive dialysis. See Appendix B, "Select and Prepare Non-Biotinylated Antibodies" on page 39.
- Perform the Forced Proximity Probe Test for *all* biotinylated antibodies, whether the antibodies are user-biotinylated or pre-biotinylated by a vendor.

Recommended Laboratory Practices and Guidelines

PCR assays require special laboratory practices to avoid false positive amplifications. The high-throughput and repetition of these assays can lead to amplification of one DNA molecule.

Good laboratory practices

When preparing samples for PCR amplification:

- Use a positive-displacement pipette or aerosol-resistant pipette tips.
- Follow proper pipette-dispensing techniques to prevent aerosols.
- Wear clean gloves and a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation).
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas and dedicated equipment and supplies for:
  - Sample preparation
  - PCR setup
  - PCR amplification
  - Analysis of PCR products
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes carefully. Centrifuge tubes before opening. Try not to splash or spray PCR samples.
- Keep reactions and components capped as much as possible.
- Clean lab benches and equipment periodically with 10% bleach solution. Use DNA*Zap*™ Solution (PN AM9890).

Pipetting guidelines

IMPORTANT! Keep all tubes and plates on ice while transferring reagents.

Precise volume delivery is crucial for the performance and reproducibility of TaqMan® Protein Assays experiments. When transferring reagents:

- Use repeat or multichannel pipettes to transfer reagents.
- Verify that all tips are properly seated prior to fluid transfer.
- Use pipettes that are calibrated regularly.
- Pre-aliquot reagents into a separate reaction plate or reservoir for loading into the multichannel pipette. Use new pipette tips for each pipetting step.
- Avoid creating bubbles when pipetting fluids.

- When preparing dilutions or reactions, place the tubes and plates on ice while transferring reagents. Use a plate holder to help stabilize the plate.
- Inspect the pipette tips to ensure consistent reagent aspiration and delivery.

Safety

This appendix covers:

Chemical safety

General chemical safety

Chemical hazard warning

WARNING! CHEMICAL HAZARD. Before handling any chemicals, refer to the Safety Data Sheet (SDS) provided by the manufacturer, and observe all relevant precautions.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical safety guidelines

To minimize the hazards of chemicals:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. (See "About SDSs" on page 56.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer's cleanup procedures as recommended in the SDS.
- Comply with all local, state/provincial, or national laws and regulations related to chemical storage, handling, and disposal.

SDSs

About SDSs

Chemical manufacturers supply current Safety Data Sheets (SDSs) with shipments of hazardous chemicals to new customers. They also provide SDSs with the first shipment of a hazardous chemical to a customer after an SDS has been updated. SDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new SDS packaged with a hazardous chemical, be sure to replace the appropriate SDS in your files.

Obtaining SDSs

The SDS for any chemical supplied by Applied Biosystems is available to you free 24 hours a day. To obtain SDSs:

1. Go to **www.appliedbiosystems.com**, click **Support**, then select **SDS**.
2. In the Keyword Search field, enter the chemical name, product name, SDS part number, or other information that appears in the SDS of interest. Select the language of your choice, then click **Search**.

3. Find the document of interest, right-click the document title, then select any of the following:
   - **Open** – To view the document
   - **Print Target** – To print the document
   - **Save Target As** – To download a PDF version of the document to a destination that you choose

Note: For the SDSs of chemicals not distributed by Applied Biosystems, contact the chemical manufacturer.

Chemical waste safety

Chemical waste hazards

CAUTION! HAZARDOUS WASTE. Refer to Safety Data Sheets and local regulations for handling and disposal.

WARNING! CHEMICAL STORAGE HAZARD. Never collect or store waste in a glass container because of the risk of breaking or shattering. Reagent and waste bottles can crack and leak. Each waste bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position. Wear appropriate eyewear, clothing, and gloves when handling reagent and waste bottles.

Chemical waste safety guidelines

To minimize the hazards of chemical waste:

- Read and understand the Safety Data Sheets (SDSs) provided by the manufacturers of the chemicals in the waste container before you store, handle, or dispose of chemical waste.
- Provide primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing). For additional safety guidelines, consult the SDS.
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with adequate ventilation (for example, fume hood). For additional safety guidelines, consult the SDS.
- Handle chemical wastes in a fume hood.
- After emptying a waste container, seal it with the cap provided.
- Dispose of the contents of the waste tray and waste bottle in accordance with good laboratory practices and local, state/provincial, or national environmental and health regulations.

Waste disposal

If potentially hazardous waste is generated, you must:

- Characterize (by analysis if necessary) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure the health and safety of all personnel in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.

IMPORTANT! Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

Biological hazard safety

General biohazard

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (**www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **www.access.gpo.gov/ nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

www.cdc.gov

Documentation and Support

Documentation

TaqMan® Protein Assays documentation

Portable document format (PDF) versions of the documents listed below are available at **www.appliedbiosystems.com**

Note: To open the PDF versions, use the Adobe Acrobat Reader software available from **www.adobe.com**

Document	Part number
TaqMan® Protein Assays Sample Prep and Assay Protocol	4449283
TaqMan® Protein Assays Sample Prep Quick Reference Card	4449771
TaqMan® Protein Assays Assay Quick Reference Card	4449281
TaqMan® Protein Assays Probe Development Protocol	4449282
TaqMan® Protein Assays Probe Development Quick Reference Card	4449772
Real-Time PCR Systems TaqMan® Protein Assays Chemistry Guide	4405780

Instrument documentation

To obtain the documents listed in this section or additional documentation, see "Obtaining support" on page 60.

7900HT/7900HT Fast system (Fast 96-Well, Standard 96-Well, or 384-Well Block Module)

Document	Part number
Applied Biosystems 7900HT Fast Real-Time PCR System Quick Reference Card: Performing Fast Gene Quantification	4351892
Applied Biosystems 7900HT Fast Real-Time PCR System Relative Quantitation Using Comparative C_T Getting Started Guide	4364016
Applied Biosystems 7900HT Fast Real-Time PCR System Absolute Quantification Getting Started Guide	4364014
Applied Biosystems 7900HT Fast Real-Time PCR System User Bulletin: Performing Fast Gene Quantification	4369584
Applied Biosystems 7900HT Fast Real-Time PCR System User Bulletin: Performing Fast Gene Quantification	4352533
Real-Time PCR Systems Chemistry Guide: Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems	4348358

7500 Fast system

Document	Part number
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Relative Quantification Getting Started Guide	4347824
Applied Biosystems 7300/7500/7500 Fast Real-Time PCR Systems Absolute Quantification Getting Started Guide	4347825
Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4387779
Applied Biosystems 7500/7500 Fast Real-Time PCR Systems Getting Started Guide for Comparative C_T/Relative Standard Curve Experiments	4387783
Real-Time PCR Systems Chemistry Guide: Applied Biosystems 7900HT Fast Real-Time PCR Systems and 7300/7500/7500 Fast Real-Time PCR Systems	4348358

StepOnePlus™ system

Document	Part number
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Reagent Guide	4379704
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Relative Standard Curve and Comparative C_T Experiments Getting Started Guide	4376785
Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems Getting Started Guide for Standard Curve Experiments	4376784

Obtaining support

For the latest services and support information for all locations, go to:

www.appliedbiosystems.com

At the Applied Biosystems web site, you can:

- Access worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.
- Search through frequently asked questions (FAQs).
- Submit a question directly to Technical Support.
- Order Applied Biosystems user documents, SDSs, certificates of analysis, and other related documents.
- Download PDF documents.
- Obtain information about customer training.
- Download software updates and patches.

Part Number 4449282 Rev. C 02/2011

applied biosystems™
by life technologies™

Headquarters
5791 Van Allen Way | Carlsbad, CA 92008 USA
Phone 760.603.7200
www.lifetechnologies.com

Technical Resources and Support
For the latest technical resources and support information for all locations, please refer to our Web site at
www.appliedbiosystems.com