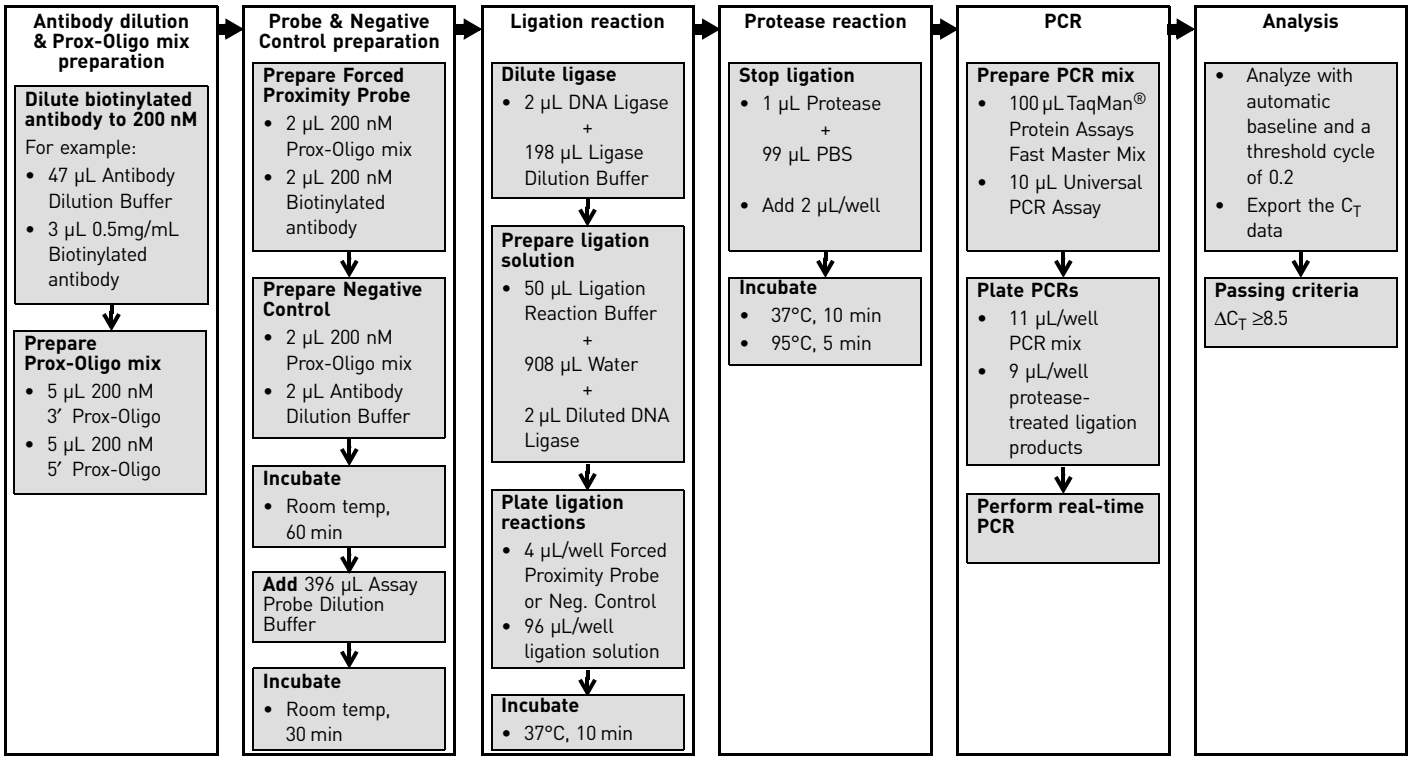
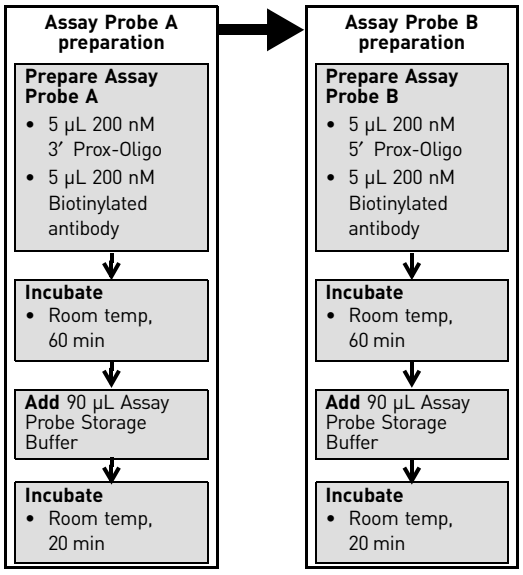
TaqMan® Protein Assays Probe Development

Note: For safety and biohazard guidelines, refer to the "Safety" section in the *TaqMan® Protein Assays Probe Development Protocol* (Part no. 4449282). For every chemical, read the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Workflow for the Forced Proximity Probe Test



Workflow for preparing Assay Probe A and Assay Probe B



Select and prepare biotinylated antibodies

Select and prepare biontinylated antibodies according to the *TaqMan®* Protein Assays *Probe Development Protocol.*

Perform the Forced Proximity Probe Test

1 Dilute the biotinylated antibody to 200 nM (30 µg/mL)

a. If you are using frozen biotinylated antibody: Remove an aliquot from storage and immediately place it on ice to thaw. After the antibody has thawed, gently mix (but do not vortex). Spin the tube briefly (~5 seconds) to bring the liquid to the tube bottom.

b. Working on ice, combine the components listed below. Briefly centrifuge to spin the liquid to the tube bottom, then place on ice.

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

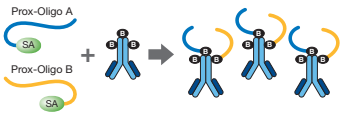
After diluting the biotinylated antibody:

- Proceed to "Prepare the Forced Proximity Probe and the negative control" below. 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 6).

2 Prepare the Forced Proximity Probe and the negative control



a. Prepare the Prox-Oligo mix: Working on ice, combine the components listed below. Mix gently, then briefly centrifuge to spin the liquid to the tube bottom. Place on ice.

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

b. Label two tubes: **Forced Proximity Probe** and **Negative Control**.

c. Working on ice, combine the components listed below in the appropriate tube. Mix gently, then briefly centrifuge to spin the liquid to the tube bottoms.

Component	Volume (µL)	
	Forced Proximity Probe	**Negative Control**
200 nM Prox-Oligo mix	2 µL	2 µL
200 nM Biotinylated antibody	2 µL	--
Antibody Dilution Buffer	--	2 µL
Total volume	**4 µL**	**4** µL

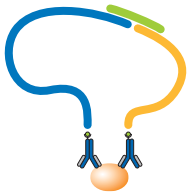
d. Incubate the tubes at room temperature for 60 minutes.

e. Add 396 µL of Assay Probe Dilution Buffer to each tube.

f. Incubate the tubes at room temperature for 30 minutes, then place both tubes on ice.

Proceed to "Perform the ligation reaction" below.

3 Perform the ligation reaction



IMPORTANT! For step 3 (performing the ligation reaction) through step 6 on page 5 (preparing the PCR plate): Keep all reagents on ice when not in use. Do not allow the tubes to warm to room temperature. Keep the reaction plates on ice during reagent transfers.

a. Thaw or place the following components on ice: 500X DNA Ligase, 1X Ligase Dilution Buffer, 20X Ligation Reaction Buffer, 1X PBS (pH 7.4), 100X Protease.

b. Dilute the DNA Ligase: Combine the components listed below. Mix gently, then place on ice.

IMPORTANT! Prepare fresh diluted ligase for each experiment.

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

c. Prepare the ligation solution: Combine the components listed below. Invert the tube to mix, then place on ice.

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

d. Place a 96-well reaction plate on ice. To 8 wells of the plate, add the components as follows:

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

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

f. Seal the ligation reaction plate with a MicroAmp® Clear Adhesive Film, then briefly centrifuge the plate.

g. Incubate the sealed reaction plate using the thermal-cycling conditions below.

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 no.	Temperature (°C)	Time	Reaction volume
Ligation	1	37°C	10 minutes	Default
Cooling	1	4°C	≤10 minutes	Default

You can omit the protease step if you proceed *immediately* to the real-time PCR steps, beginning with "Prepare the PCR mix" on page 5. Otherwise, continue with the protease reaction below.

4 Perform the protease reaction

a. Dilute the protease:

1. Briefly vortex the protease to mix the solution.
2. Combine the components listed below. Mix gently, then briefly centrifuge to spin the liquid to the tube bottom. Place on ice.

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

b. Remove the ligation reaction plate from the thermal cycler, remove the MicroAmp Clear Adhesive Film, 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.

d. Reseal the reaction plate with a new adhesive film.

e. Incubate the sealed reaction plate using the thermal-cycling conditions below.

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°C	10 minutes	Default
Inactivate protease	1	95°C	5 minutes	Default
HOLD	1	4°C	Hold	Default

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

Proceed to "Prepare the PCR mix" on page 5 OR store the protease-treated ligation products at 4°C for up to 3 days, or at –20°C for up to 2 weeks.

5 Prepare the PCR mix

a. Thaw the Universal PCR Assay on ice.

b. Combine the components listed below. Mix gently, then briefly centrifuge to spin the liquid to the tube bottom. Place on ice.

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

6 Prepare the PCR plate

a. Place a PCR plate on ice, then add 11 µL of the PCR mix to each of 8 wells of the PCR plate.

b. Remove the adhesive film from the protease reaction plate, then place the plate on ice.

c. 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.

d. Seal the PCR plate with a MicroAmp® Optical Adhesive Film, then briefly centrifuge the plate. For the 7900HT/7900HT Fast system with a 96-Well Block Module and automation accessory, place a MicroAmp® Snap-On Optical Film Compression Pad on top of the plate.

IMPORTANT! Proceed *immediately* to "Run the PCR plate" below.

7 Run the PCR plate

In your real-time PCR system software, create a plate document/experiment for the run, using the setup information below. Load the PCR plate into your real-time PCR instrument, then start the run.

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				

8 Analyze the data

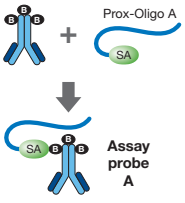
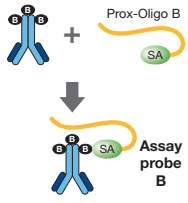
Method	Procedure
Using the real-time PCR system software	1. View the amplification plots for the entire reaction plate. 2. Analyze the plate run using a threshold cycle (C_T) setting of 0.2 and automatic baseline.
Using a spreadsheet program	1. Export the results from the instrument software to a spreadsheet program. 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	ΔC_T value	Result	Comment
	≥8.5	Pass	The biotinylated antibody is suitable for use in TaqMan® Protein Assays experiments.

Prepare Assay Probe A or Assay Probe B

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.

1. Briefly centrifuge the 200 nM biotinylated antibody to spin the liquid to the tube bottom, then place the tube on ice.
2. Briefly centrifuge the appropriate Prox-Oligo to spin the liquid to the tube bottom, then place the tube on ice. Use the:
   - 200 nM 3′ Prox-Oligo for Assay Probe A
   - 200 nM 5′ Prox-Oligo for Assay Probe B
3. Working on ice, combine the components listed below.

Component	Volume (µL)			
	For Assay Probe A		For Assay Probe B	
200 nM Biotinylated antibody	5		5	
200 nM 3′ Prox-Oligo	5		NA	
200 nM 5′ Prox-Oligo	NA		5	
Total volume	**10**		**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 the Assay Probe at –20°C for up to 6 months.

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