

Designing Assays for the TaqMan® Protein Assay

Antibody Selection	2
Assay Design.....	2
Antibody Search	3
Biotinylated Antibodies	3
Working with pre-biotinylated antibodies.....	4
Working with non-biotinylated antibodies.....	5
Forced Proximity Probe Test	7
About the Forced Proximity Probe Test	7
Expected Results and Troubleshooting Guide.....	7
Assay Optimization.....	8
Assay Probe Preparation	8
Assay Probe Concentration in Binding Reaction	8
Binding Reaction Time and Temperature	9
Binding Buffer	9
Sample Preparation	9
Use of Controls	10
TaqMan® Protein Assay - general workflow.....	10
Positive Assay for use with your lysates from human cells or tissues.....	10
Positive Assay for use with your lysates from mouse embryonal stem cells or equivalent	11
Positive Assay for use with your lysates from normal mouse cells or tissues	11
Preparing Assay Probes with the Open Kit and your own biotinylated antibody(ies)	12
Preparing Assay Probes with the Open Kit and your own non-biotinylated antibody(ies)	12
Negative Control – Mismatched Assay Probes	13
For More Information.....	14
Legal Acknowledgements	15

Antibody Selection

Assay Design

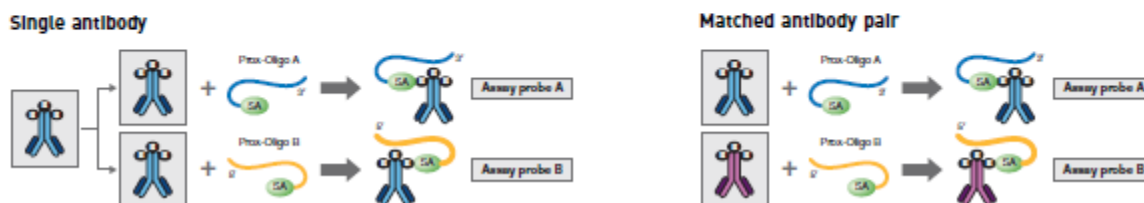
TaqMan[®] Protein Assays can be developed for a wide variety of targets with user-supplied biotinylated antibodies and the TaqMan[®] Protein Assays Open Kit. The kit contains 5' and 3' Prox-Oligos (streptavidin-linked oligos) and buffers required to make the two Assay Probes used for binding in the TaqMan[®] Protein Assay. The TaqMan[®] Protein Assay requires antibodies that bind to two or more distinct epitopes on the target protein to enable ligation of the 3' and 5' oligos. Depending on the availability of antibodies to your chosen target, there may be several assay design options.

The simplest assay design uses a single polyclonal antibody that is split into two pools to generate the 3' and 5' oligo-linked antibodies (Assay Probes A and B) (see figure 1). It is important, however, that the polyclonal antibody is raised against a full length or near-full length antigen (>100 aa). An Assay Probe pair made with a polyclonal antibody raised against a small peptide (<100 aa) will probably not be successful in the TaqMan[®] Protein Assay since it is likely that the antibody would bind to only a single epitope. Note that a single monoclonal antibody is also not suitable for generating a pair of Assay Probes because it, too, binds to a single epitope and cannot form the requisite proximity binding pair.

An alternative assay design uses two different antibodies that have been qualified to function as a match pair in an ELISA. The matched pair can be polyclonal/monoclonal, monoclonal/monoclonal, or polyclonal/polyclonal, or (see figure 2).

It is important that the polyclonal antibodies used to make the Assay Probes are antigen purified. Non-antigen purified antibodies usually contain significant amounts of non-specific antibodies and their presence in the TaqMan[®] Protein Assay would diminish the sensitivity and specificity of the assay.

	Description	Assay Probe pair configuration
Antigen-purified polyclonal	Raised against full length or near full length antigen (>100 aa)	Single antibody or matched antibody pair
	Raised against peptide (<100 aa)	Matched antibody pair
Monoclonal		Matched antibody pair



Antibody Search

Due to the inherent nature of antibodies, Applied Biosystems cannot guarantee that the antibody or antibody pair that you select will result in a working proximity probe set. Refer to the *TaqMan® Protein Assays Probe Development Protocol* 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. The overall success rate with these antibodies is 74%. To view the most current list, go to: www.appliedbiosystems.com/taqman4antibodies. If you cannot find your target in the list of positively tested assays, and you do not yet have antibodies to try in the TaqMan Protein Assay, you may be able to find commercially available antibodies to your target by searching online at www.labome.com and www.linscottsdirectory.com.

Biotinylated Antibodies

The Assay Probes are prepared with biotinylated antibodies. If the antibody you select is not biotinylated, you must label the antibody with biotin and remove unreacted biotin using dialysis. The TaqMan® Protein Assay is very sensitive to free biotin. As little as 80 nM of free biotin in the antibody preparation will diminish assay performance (see Appendix C, “*Example Forced Proximity Probe Test and TaqMan® Protein Assays Data.*” in the *TaqMan® Protein Assays Probe Development Protocol*). Consequently, biotinylated antibody preparations must not contain any free biotin. It is strongly recommended that you perform the Forced Proximity Probe Test for all newly purchased or made biotinylated antibodies. (see Forced Proximity Probe Test section). Requirements for pre-biotinylated and non-biotinylated antibodies are summarized in the table below.

Antibody	Requirements	Recommended kits for biotinylation and dialysis	Forced Proximity Probe Test
Pre-biotinylated	Does not contain any free biotin	NA	Must pass the Forced Proximity Probe Test ($\Delta CT > 8.5$). See “Forced Proximity Probe Test” section.
Non-biotinylated	Free of carrier protein and in an amine-free buffer (for example, no BSA, gelatin, glycine, Tris, or azide).	<ul style="list-style-type: none"> • EZ-Link® Sulfo-NHS-LC-Biotin, No-Weigh™ Format (Pierce, PN 21327) or • Biotin-XX Microscale Protein Labeling Kit (Molecular Probes, PN B30010) 	
	Requires thorough dialysis to remove free biotin after the biotinylation reaction.	Slide-A-Lyzer® Mini Dialysis Unit (MWCO=7000) (Thermo Scientific, PN 69562)	

Working with pre-biotinylated antibodies

If the pre-biotinylated antibody is obtained in lyophilized form, follow the detailed protocol in the *Resuspend Lyophilized Biotinylated Antibodies* section of the *TaqMan® Protein Assays Probe Development Protocol*. The workflow is as follows:

1. Resuspend pre-biotinylated antibody in Antibody Dilution Buffer
2. Place the biotinylated antibody stock solution on ice and proceed *immediately* to “Perform the Forced Proximity Probe Test”

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.

3. If the pre-biotinylated antibody does not pass the test (passing criteria $\Delta C_t > 8.5$), then dialyze the antibody (see below) and repeat the Forced Proximity Probe Test.
4. 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”

If the pre-biotinylated antibody is already in solution, then follow step 2 (first part) to step 4.

Required Reagents	Part Number
TaqMan® Protein Assays Open Kit	4453745
TaqMan® Protein Assays Core Reagents Kit with Master Mix	4405501

Working with non-biotinylated antibodies

There are some options available if the antibody you choose is not already biotinylated. Inquire whether the antibody vendor offers custom services or an off-catalogue biotinylated version of the chosen antibody. Alternatively, you may biotinylate the antibody yourself. The antibody preparation must meet the following requirements for biotinylation:

- Be in an amine-free buffer, such as 1x PBS (for example, no glycine, Tris or azide)
- Contain no carrier protein (for example, BSA or gelatin)

If the antibody preparation is in an amine-containing buffer, it may be dialyzed in 1x PBS pH 7.4 to exchange the buffer. If the antibody preparation contains carrier protein, the carrier protein must be removed prior to biotinylation. Several vendors (including Invitrogen and Pierce) offer antibody purification kits for different types of antibodies.

If the non-biotinylated antibody is obtained in lyophilized form, follow the detailed protocol in the *Select and Prepare Non-Biotinylated Antibodies* section of the *TaqMan[®] Protein Assays Probe Development Protocol*. The workflow is as follows:

1. Resuspend non-biotinylated antibody in 1x PBS pH 7.4
2. Label the antibody with biotin.
3. Remove free biotin using extensive dialysis (column chromatography is not recommended).
 - (Recommended) Use the Slide-A-Lyzer[®] Mini Dialysis Unit.
 - Perform dialysis at 4 °C in cold 1X PBS, pH 7.4 (user-supplied). Change the buffer at least five times, as follows:

Dialysis parameters

No. of buffer changes	Interval time	Volume (mL) of 1X PBS
Four	2 hours	500
One	Overnight [†]	1000

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

4. Place the biotinylated antibody stock solution on ice and proceed *immediately* to “Perform the Forced Proximity Probe Test”

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.

5. If the biotinylated antibody does not pass the test (passing criteria $\Delta C_t > 8.5$), then re-dialyze the antibody and repeat the Forced Proximity Probe Test.
6. After the biotinylated antibody has passed the Forced Proximity Probe Test, you can aliquot the stock solution for long-term storage at -80 °C after adding carrier protein to 0.1% (1 mg/mL). See “Storing the biotinylated antibody after the Forced Proximity Probe Test”

If the non-biotinylated antibody is already in solution, then follow step 2 to step 6.

Required Reagents	Vendor	Part Number
Slide-A-Lyzer® Mini Dialysis Unit (MWCO=7000)	Thermo Scientific	69562
Biotin-XX Microscale Protein Labeling Kit	Molecular Probes	B30010
or		
EZ-Link® Sulfo-NHS-LC-Biotin, No-Weigh™ Format	Pierce	2132
TaqMan® Protein Assays Open Kit		4453745
TaqMan® Protein Assays Core Reagents Kit with Master Mix		4405501

Forced Proximity Probe Test

About the Forced Proximity Probe Test

The Forced Proximity Probe Test determines whether or not the biotinylated antibody is suitable for making proximity probes. The test will fail if free biotin is in the preparation or if the antibody is under-biotinylated. The test will also fail if your biotinylated antibody is less concentrated than expected. Applied Biosystems recommends that you perform the Forced Proximity Probe Test for *all* biotinylated antibodies, whether the antibodies are user-biotinylated or pre-biotinylated by a vendor. It is important to note that 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.

Expected Results and Troubleshooting Guide

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.

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	<ul style="list-style-type: none"> • 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	<p>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.</p> <p>If the concentration is not correct, repeat the Forced Proximity Probe Test with the correct amount of biotinylated antibody.</p> <p>If the concentration is correct:</p> <ol style="list-style-type: none"> 1. Redialyze the biotinylated antibody for an additional 1 to 2 buffer changes. 2. Perform the Forced Proximity Probe Test again. <p>If the biotinylated antibody fails the Forced Proximity Probe Test a second time, the antibody may not be biotinylated. Make sure there are no primary amines (e.g. Tris, azide or glycine) in the antibody preparation. Prepare or obtain more biotinylated antibody, then perform the Forced Proximity Probe Test again.</p>

Assay Optimization

The TaqMan® Protein Assay can be optimized at several stages of the protocol. The following describes modifications to the protocol that may be used to improve the performance of your TaqMan® Protein Assay.

Assay Probe Preparation

The following variation on the Assay Probe preparation protocol uses less Prox-Oligos and may improve the signal/noise of the assay (see *Prepare the Assay Probes* in the *TaqMan® Protein Assays Probe Development Protocol*). The remainder of the Assay Probe preparation protocol is unchanged (final volume of Assay Probes is 100 µL). This variation is suggested for use only with polyclonal antibodies.

Assay Probe A

Component	Standard Protocol Volume (µL)	Modified Protocol Volume (µL)
200 nM Biotinylated antibody	5	5
Antibody Dilution Buffer	NA	2.5
200 nM 3'Prox-Oligo	5	2.5
Final	10	10

Assay Probe B

Component	Standard Protocol Volume (µL)	Modified Protocol Volume (µL)
200 nM Biotinylated antibody	5	5
Antibody Dilution Buffer	NA	2.5
200 nM 5'Prox-Oligo	5	2.5
Final	10	10

Assay Probe Concentration in Binding Reaction

The concentration of the Assay Probes in the binding reaction may be reduced in an effort to improve the signal/noise. The concentration of each Assay Probe in the standard binding reaction is 250 pM. It is not advisable to have less than 50 pM of each Assay Probe in the binding reaction as the background signal (noise) of the assay will be too variable. The following table provides suggested reduced Assay Probe A and B volumes to make solutions with reduced Assay Probe concentrations (see *Perform the binding reaction* in the *TaqMan® Protein Assays Sample Prep and Assay Protocol*)

Order to combine	Component	Standard volumes	Modified volumes			
1	Assay Probe Dilution Buffer, 1X	216 µL	220.8 µL	225.6 µL	230.4 µL	235.2 µL
2	Assay Probe A (10 nM)	12 µL	9.6 µL	7.2 µL	4.8 µL	2.4 µL
3	Assay Probe B (10 nM)	12 µL	9.6 µL	7.2 µL	4.8 µL	2.4 µL
Total volume of Assay Probe solution		240 µL	240 µL	240 µL	240 µL	240 µL
Concentration of each Assay Probe in Assay Probe solution		500 pM	400 pM	300 pM	200 pM	100 pM
Concentration of each Assay Probe in binding reaction		250 pM	200 pM	150 pM	100 pM	50 pM

Binding Reaction Time and Temperature

The standard binding reaction is at 37°C for 60 minutes. However, the signal/noise for your assay may be improved by modifying the time and/or temperature of the binding reaction. Suggested alternate binding temperatures and intervals include 25°C and 4°C and from several hours to overnight, respectively.

Binding Buffer

The standard buffer used in the binding reaction is either the Lysate Dilution Buffer or the Cell Resuspension Buffer, depending on which TaqMan® Protein Assay kit is used. Note that the buffers are essentially the same and are interchangeable. The standard buffer is PBS-based with 0.1% carrier protein. To improve the signal/noise of your assay, you may decide to include additives such as extra carrier protein (e.g. gelatin or nonfat powdered milk). The ligation and qPCR reactions are not inhibited with binding reactions containing up to 0.5% gelatin or 5% nonfat powdered milk (the use of BSA is not recommended).

Sample Preparation

It is possible to improve the signal/noise by modifying your sample prep procedure and/or by using another lysis reagent. Appendix A in the TaqMan® Protein Assays Sample Prep and Assay Protocol lists alternate lysis reagents for cells and tissues that are known to be compatible with the TaqMan® Protein Assay. Additional treatments, such as lysate clarification by centrifugation or heat treatment (i.e. 75°C for 10 minutes) may also help improve assay performance.

Use of Controls

The use of controls is very helpful when developing a new TaqMan[®] Protein Assay and preparing new Assay Probes with the Open Kit. The following lists the different controls that may be used at each stage of the TaqMan[®] Protein Assay workflow.

TaqMan[®] Protein Assay - general workflow

If you are new to TaqMan[®] Protein Assays, it is recommended that you familiarize yourself with the workflow using a control lysate (Raji) and pre-made Assay Probes (hCSTB).

Reagent	Part Number
TaqMan [®] Protein Assay Kit (hCSTB)	4405465
Protein Expression Lysate Control Kit (Raji)	4405448
TaqMan [®] Protein Assays Core Reagents Kit with Master Mix	4405501

Positive Assay for use with your lysates from human cells or tissues

CSTB is ubiquitously expressed in most tissue or cell types and can serve as a positive control with lysates that you generate from human cells or tissues. Note that CSTB should not be used as an endogenous control for relative quantification purposes. It is advisable to include the Raji lysate as the positive control lysate.

Reagent	Part Number
TaqMan [®] Protein Assay Kit (hCSTB)	4405465
Protein Expression Lysate Control Kit (Raji)	4405448
TaqMan [®] Protein Assays Core Reagents Kit with Master Mix	4405501

Sample Prep Reagent	Part Number
Protein Quant Sample Lysis Kit	4448536
or	
Protein Expression Sample Prep Kit	4405443

Positive Assay for use with your lysates from mouse embryonal stem cells or equivalent

Applied Biosystems does not have pre-made Assay Probes to mouse targets. However, the hSOX2 Assay Probes will moderately cross react with mSOX2. It may be possible, therefore to detect moderate to high levels of mSOX2 in mouse cells or tissues expressing mSOX2. The hSOX2 Assay Probes have successfully detected mSOX2 in P19 [ATCC CRL-1825] and F9 [ATCC CRL-1720] lysates.

Reagent	Part Number
TaqMan [®] Protein Assay Kit (hSOX2)	4405495
TaqMan [®] Protein Assays Core Reagents Kit with Master Mix	4405501

Sample Prep Reagent	Part Number
Protein Quant Sample Lysis Kit	4448536
or	
Protein Expression Sample Prep Kit	4405443

Positive Assay for use with your lysates from normal mouse cells or tissues

We have made mICAM1 Assay Probes using the Open Kit and a biotinylated polyclonal mICAM1 antibody and successfully detected mICAM1 in a mouse universal tissue lysate. The mICAM1 assay may therefore be a suitable positive control for use with your mouse cell or tissue lysates. This positive control requires that you prepare your own mICAM1 Assay Probes with the Open Kit and the commercially available biotinylated polyclonal mICAM1 antibody. The mouse universal tissue lysate also serves as a useful positive control.

Reagent	Vendor	Part Number
Biotinylated Anti-mouse ICAM-1 Antibody	R&D Systems	BAF796
Universal Protein Lysate: Mouse Normal Tissues	BioChain	P4334566
TaqMan [®] Protein Assays Open Kit		4453745
TaqMan [®] Protein Assays Core Reagents Kit with Master Mix		4405501

Sample Prep Reagent	Part Number
Protein Quant Sample Lysis Kit	4448536
or	
Protein Expression Sample Prep Kit	4405443

Preparing Assay Probes with the Open Kit and your own biotinylated antibody(ies)

When making Assay Probes with your own biotinylated antibody or antibody pair, it may be useful to generate an Assay Probe with a control biotinylated antibody to serve as a process control. The pre-made hCSTB Assay Probes from Applied Biosystems are prepared with a biotinylated anti-human CSTB polyclonal antibody from R&D Systems. You can prepare hCSTB Assay Probes with the biotinylated antibody and compare the performance of your hCSTB Assay Probes with the pre-made hCSTB Assay Probes from Applied Biosystems using the Raji lysate control.

Reagent	Vendor	Part Number
Biotinylated Anti-human CSTB Antibody	R&D Systems	BAF1408
TaqMan [®] Protein Assays Open Kit		4453745
TaqMan [®] Protein Assay Kit (hCSTB)		4405465
Protein Expression Lysate Control Kit (Raji)		4405448
TaqMan [®] Protein Assays Core Reagents Kit with Master Mix		4405501

Preparing Assay Probes with the Open Kit and your own non-biotinylated antibody(ies).

You will need to biotinylate your antibody or antibody pair before making the Assay Probes. It may be useful to biotinylate a control antibody to serve as a process control. You can biotinylate the hCSTB antibody from R&D Systems and prepare Assay Probes with your biotinylated antibody, as well as with the biotinylated version of the same antibody. You can then compare the performance of those Assay Probes with the pre-made hCSTB Assay Probes from Applied Biosystems using the Raji lysate control.

Reagent	Vendor	Part Number
Anti-human CSTB Antibody	R&D Systems	AF1408
Biotinylated Anti-human CSTB Antibody	R&D Systems	BAF1408
Slide-A-Lyzer [®] Mini Dialysis Unit (MWCO=7000)	Thermo Scientific	69562
Biotin-XX Microscale Protein Labeling Kit	Molecular Probes	B30010
or		
EZ-Link [®] Sulfo-NHS-LC-Biotin, No-Weigh [™] Format	Pierce	2132
TaqMan [®] Protein Assays Open Kit		4453745
TaqMan [®] Protein Assay Kit (hCSTB)		4405465
Protein Expression Lysate Control Kit (Raji)		4405448
TaqMan [®] Protein Assays Core Reagents Kit with Master Mix		4405501

Negative Control – Mismatched Assay Probes

A negative control should always be included in your experimental design. It is best to use a sample that is known not to express the target of interest as your negative control. However, if no such sample exists, or to use an additional negative control you can use a mismatched Assay Probe pair. If, for example, your target is hCSTB, you can use a mismatched hCSTB/hICAM1 Assay Probe pair (hCSTB Assay Probe A + hICAM1 Assay Probe B). The mismatched Assay Probe Pair should only generate background signal since both antibodies will not bind to the same target to enable proximity ligation of the 5' and 3' oligos.

Antibody Selection

Assay Design

TaqMan[®] Protein Assays can be developed for a wide variety of targets with user-supplied biotinylated antibodies and the TaqMan[®] Protein Assays Open Kit. The kit contains 5' and 3' Prox-Oligos (streptavidin-linked oligos) and buffers required to make the two Assay Probes used for binding in the TaqMan[®] Protein Assay. The TaqMan[®] Protein Assay requires antibodies that bind to two or more distinct epitopes on the target protein to enable ligation of the 3' and 5' oligos. Depending on the availability of antibodies to your chosen target, there may be several assay design options.

The simplest assay design uses a single polyclonal antibody that is split into two pools to generate the 3' and 5' oligo-linked antibodies (Assay Probes A and B) (see figure 1). It is important, however, that the polyclonal antibody is raised against a full length or near-full length antigen (>100 aa). An Assay Probe pair made with a polyclonal antibody raised against a small peptide (<100 aa) will probably not be successful in the TaqMan[®] Protein Assay since it is likely that the antibody would bind to only a single epitope. Note that a single monoclonal antibody is also not suitable for generating a pair of Assay Probes because it, too, binds to a single epitope and cannot form the requisite proximity binding pair.

An alternative assay design uses two different antibodies that have been qualified to function as a match pair in an ELISA. The matched pair can be polyclonal/monoclonal, monoclonal/monoclonal, or polyclonal/polyclonal, or (see figure 2).

It is important that the polyclonal antibodies used to make the Assay Probes are antigen purified. Non-antigen purified antibodies usually contain significant amounts of non-specific antibodies and their presence in the TaqMan[®] Protein Assay would diminish the sensitivity and specificity of the assay.

	Description	Assay Probe pair configuration
Antigen-purified polyclonal	Raised against full length or near full length antigen (>100 aa)	Single antibody or matched antibody pair
	Raised against peptide (<100 aa)	Matched antibody pair
Monoclonal		Matched antibody pair

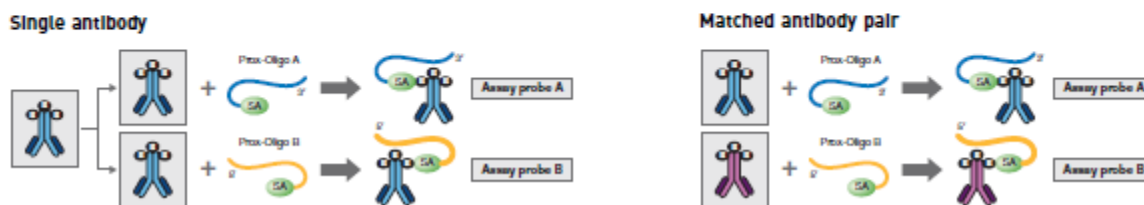


Figure 1

Figure 2

Legal Acknowledgements

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