

lifetechnologies社

## リアルタイムPCR(PTS)セミナー

【セミナー資料】

2013年5月24日(金)

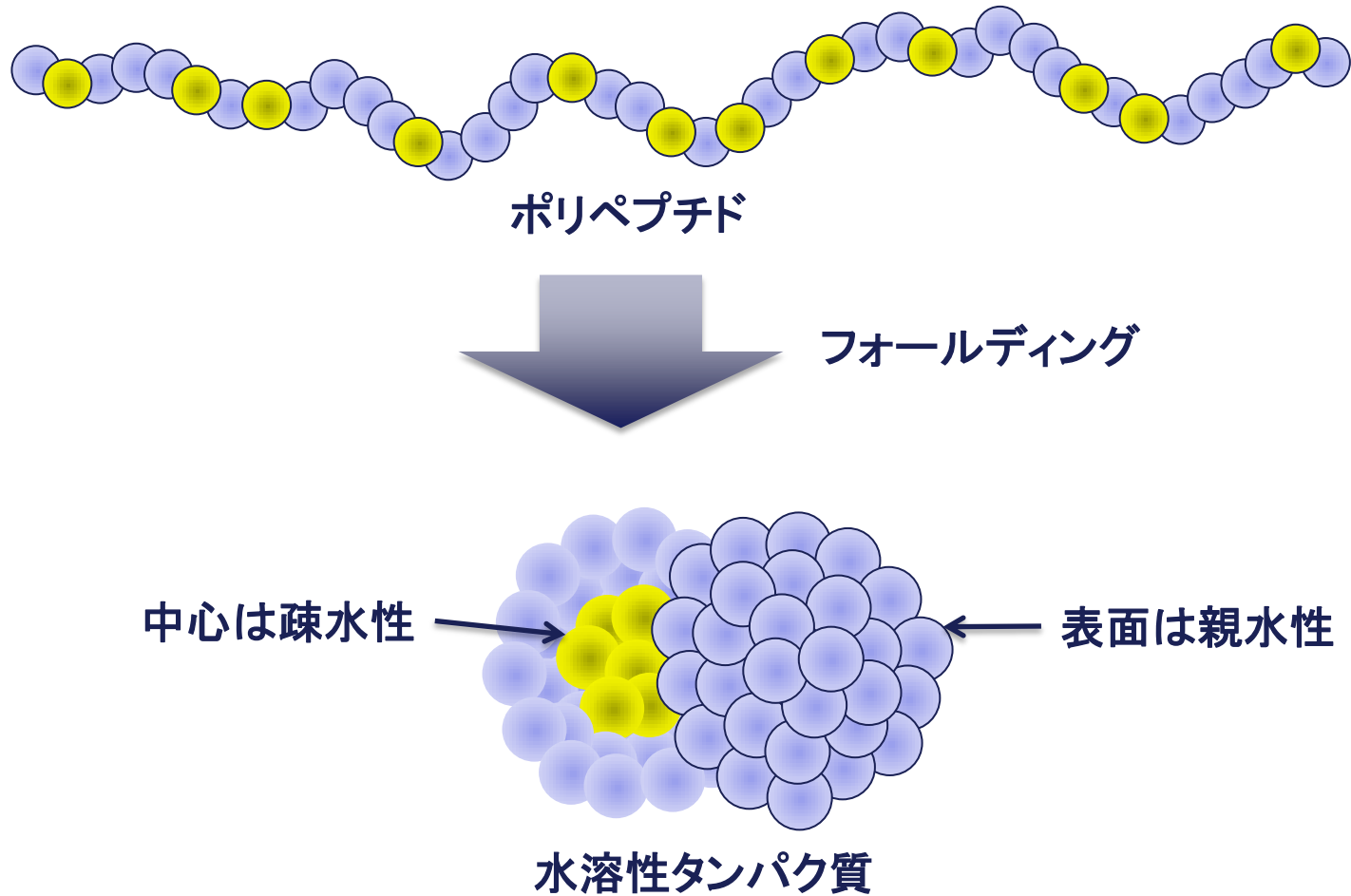
- 資料の学内限定公開についてはlifetechnologies株式会社の許可を得ておりますが、資料および音声データの二次配布については固く禁止させていただきます
- 資料内部に記載されている消耗品価格は当時のものであり、現在は変更されている可能性がありますので、価格については各自でご確認下さい

# リアルタイムPCR を用いた タンパク質のスクリーニング

ライフテクノロジーズジャパン株式会社  
テクニカルサポート

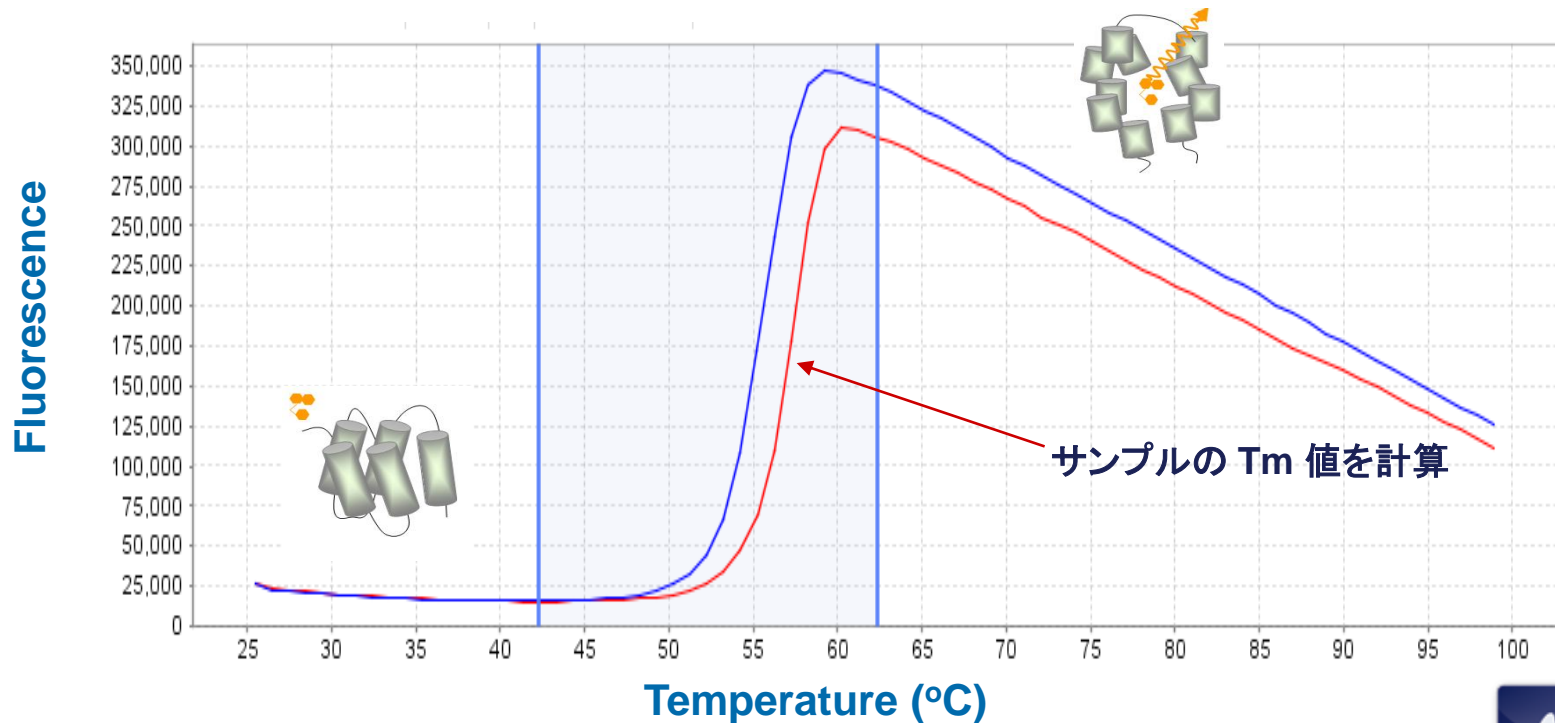


# 水溶性タンパク質と疎水性コア



# Protein Thermal Shift™ の原理

- 水溶液中のタンパク質が変性すると、タンパク質の疎水性領域が露出する
- 蛍光物質がタンパク質の疎水性領域に結合し、蛍光を発する
- 温度を上昇させながら蛍光量を測定し、タンパク質の融解曲線を作成する
- タンパク質の  $T_m$  値はタンパク質の安定性と関連している
- タンパク質の最適バッファの検討、Ligand のスクリーニングなどに適応可



# 実験手順

- ①リアルタイムPCRで融解実験  
(蛍光データ取得)



- ②実験ファイルをインポートして解析



1000X の Dye を希釈し  
8X の溶液を調整



## 反応液調整例

Component	Volume
Protein Thermal Shift™ Buffer	5.0 $\mu$ L
Water + 2.0 $\mu$ L Protein Thermal Shift™ Control Protein + Protein Thermal Shift™ Control Ligand (0 mM, 0.1 mM, or 1 mM final concentration)	12.5 $\mu$ L
Diluted Protein Thermal Shift™ Dye (8X)	2.5 $\mu$ L
<b>Total volume for each control reaction</b>	<b>20.0 <math>\mu</math>L</b>

タンパク質量 (0.05~5 $\mu$ g / well)  
1 $\mu$ g / well が標準的



# Experiment property

Experiment Menu << Experiment: **Untitled 1** Type: **Melt Curve** Reagents: **Other** **START RUN** ?

**Setup**

**Experiment Properties**

Plate Setup

Run Method

Materials List

**Run**

**Analysis**

**Experiment Properties**

How do you want to identify this experiment?

\* Experiment Name:

Barcode (Optional):

User Name (Optional):

Comments (Optional):

**機種を選択**

\* Which instrument are you using to run the experiment?

StepOnePlus™ Instrument (96 Wells)  StepOne™ Instrument (48 Wells)

Set up, run, and analyze an experiment using a 4-color, 96-well system.

\* What type of experiment do you want to set up?

Quantitation - Standard Curve  Quantitation - Relative Standard Curve  Quantitation - Comparative Ct ( $\Delta\Delta Ct$ )

**Melt Curve**  Presence/Absence

Determine the melting temperature of a target nucleic acid sequence and identify nonspecific PCR amplification.

\* Which reagents do you want to use to detect the target sequence?

SYBR® Green Reagents  **Other**

The PCR reactions contain primers to designed to amplify the target sequence and other reagents to detect amplification. The Reaction Setup screen is not available for "Other" reagents.

Include Melt Curve

\* Which ramp speed do you want to use in the instrument run?

Standard (~ 2 hours to complete a run)  **Fast (~ 40 minutes to complete a run)**

**どちらでも構いません**

# Plate Setup ( Define Targets and Samples )

StepOne™ Software v2.2.2  
File Edit Instrument Analysis Tools Help

New Experiment Open Save Close Send Experiment to Instrument... Download Experiment from Instrument... Export...

Experiment Menu << Experiment: 110801PTS\_control Type: Melt Curve Reagents: Other

Define Targets and Samples Assign Targets and Samples

Instructions: Define the targets to quantify and the samples to test in the reaction plate.

Define Targets

Add New Target Add Saved Target Save Target Delete Target

Target Name	Reporter	Quencher	Color
T4Lig	ROX	None	Blue
T4Lig Ligand	ROX	None	Green
NPC	ROX	None	Pink
LOC	ROX	None	Orange

Define Samples

Add New Sample Add Saved

Sample Name

Sample 1

Add New Targetからターゲットを加えTarget Nameを指定  
(Reporter : ROX Quencher : None )

注:この設定はProtein Thermal Shift softwareには反映されない  
ので必須ではない

# Plate Setup ( Assign Targets and Samples )

StepOne™ Software v2.2.2

File Edit Instrument Analysis Tools Help

New Experiment Open Save Close Send Experiment to Instrument Download Experiment from Instrument Export Print Report

Experiment: 110801PTS\_control Type: Melt Curve Reagents: Other **START RUN**

Experiment Menu << **Setup** Experiment Properties **Plate Setup** Run Method Materials List Run Analysis

Define Targets and Samples **Assign Targets and Samples**

**Instructions:** To set up unknowns: Select wells, assign target(s), select "U" (Unknown) as the task for each target assignment, then assign a sample. To set up negative controls: Select wells, assign target(s), then select "N" (Negative Control) as the task for each target assignment.

**Assign Target(s) to the Selected Wells.**

Assign	Target	Task
<input checked="" type="checkbox"/>	T4Lig	<input type="button" value="U"/> <input type="button" value="N"/>
<input type="checkbox"/>	T4Lig Ligand	<input type="button" value="U"/> <input type="button" value="N"/>
<input type="checkbox"/>	NPC	<input type="button" value="U"/> <input type="button" value="N"/>
<input type="checkbox"/>	LOC	<input type="button" value="U"/> <input type="button" value="N"/>

\* Mixed  Unknown  Negative Control

**Assign Sample to the Selected Wells.**

Assign	Sample
<input type="checkbox"/>	Sample 1

Select the dye to use as the passive reference.  
 **None**

**View Plate Layout** View Well Table

Select Wells With: -Select Item - -Select Item -

Show in Wells View Legend

	1	2	3	4	5	6	7	8
A	<input type="button" value="U"/> T4Lig	<input type="button" value="U"/> T4Lig	<input type="button" value="U"/> T4Lig					
B	<input type="button" value="U"/> T4Lig Lig...	<input type="button" value="U"/> T4Lig Lig...	<input type="button" value="U"/> T4Lig Lig...					
C	<input type="button" value="U"/> NPC	<input type="button" value="U"/> NPC	<input type="button" value="U"/> NPC					
E								
F								

Wells:  12 Unknown  0 Negative Control **36 Empty**

反応液を含むウェルを選択し  
TargetのAssignチェックボックスに✓を入れる



# Run Method

The screenshot displays the StepOne Software v2.2.2 interface. The top menu bar includes File, Edit, Instrument, Analysis, Tools, and Help. Below the menu bar, there are buttons for New Experiment, Open, Save, Close, Send Experiment to Instrument, Download Experiment from Instrument, Export, and Print Report. The main window is titled "Experiment: Untitled 1" and "Type: Melt Curve". The "Run Method" section is highlighted in the left sidebar. The "Run Method" panel shows a graphical view of the thermal profile. The profile consists of two steps: Step 1 (25.0 °C, 02:00) and Step 2 (99.0 °C, 02:00). The "Continuous" option is selected for Step 2. The "Save Run Method ..." button is highlighted in green. The "Reaction Volume Per Well" is set to 20 µL. The "Melt Curve Stage" is set to "Continuous".

Experiment: **Untitled 1**      Type: **Melt Curve**      Reagents: **Other**      **START RUN**

**Run Method**

Review the reaction volume and the thermal profile for the default run method. If needed, edit the default run method or select a run method from the library.

Graphical View    Tabular View

Reaction Volume Per Well: 20 µL

Add Stage ▼   Add Step ▼   Delete Selected   Undo "Set Temperature" (nothing to Redo)   Collect Data ▼   Open Run Method   **Save Run Method ...**

Melt Curve Stage

Continuous    Step and Hold

**Continuous**      **99°C2分**

100      99.0 °C  
75      02:00  
50      1%  
25      25.0 °C  
0      100%      02:00

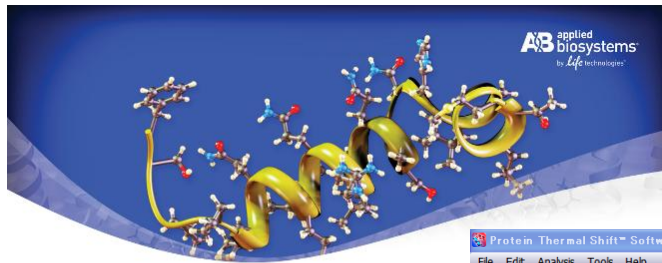
Step 1      Step 2

Legend

Data Collection On   Data Collection Off   ▲ AutoDelta On   ▲ AutoDelta Off

Save Run Methodより設定保存可能  
Open Run Methodで読み込み

# Protein Thermal Shift™ Software起動



Applied Biosystems®  
Protein Thermal Shift™ Software v1.0

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## 新規Studyの作成

Study Name	Description	# of Experiments	Date Created	Created by	Last Modified
Multi peak		1	2011-04-14 01:51:49 JST		2011-04-27 04:09:34 JST
Mutant Screen Demo		1	2011-04-27 04:04:33 JST		2011-04-27 04:05:36 JST
Buffer Screen Demo		1	2011-04-27 04:01:28 JST		2011-04-27 04:04:28 JST
Ligand Test Demo		1	2011-04-27 03:41:04 JST		2011-04-27 04:01:22 JST

**Create Study**

Studyとは同一条件で行われた実験のまとまりで  
複数枚の反応プレートで行われた実験(ファイル)を含むことができる



# 各ウェルの設定

Protein Thermal Shift™ Software Version 1.0

File Edit Analysis Tools Help

Save Save as Close Analyze Analysis Settings Export Help

Workflow Menu

Setup

Properties

Conditions

**Experiment Files**

Analysis

Export

Experiment Files

Import Delete Generate Plate Template Load Plate Template

Plate Barcode	# of Wells	Description
	48	

①設定するWellを選択する

②Assignから項目、条件を選択→Done

Plate Setup for: 110801PTS\_controlLeds

Assign Auto-Fill Settings Show in Well Color by: Ligand Legend

	1	2	3	4	5	6
A						
B						
C						
D						

Done Cancel

Protein: [ ] [ ]

Ligand: Ligand 0mM [ ] [ ]

Buffer: [ ] [ ]

Salt: [ ] [ ]

Task: Sample [ ]

Analysis Group: AG 1 [ ] [ ]

③(オプション)  
基準サンプルはTaskをReference  
No Protein ControlはNPC

# 解析

Protein Thermal Shift™ Software Version 1.0

File Edit Analysis Tools Help

Save Save as Close Analyze Analysis Settings Export Help

Workflow Menu

- Setup
- Properties
- Conditions
- Experiment Files**
- Analysis
- Export

### Experiment Files

Import Delete Generate Plate Template Load Plate Template

Experiment File Name	Color	Plate Barcode	# of Wells	Description
110801PTS_control.eds	Red		48	

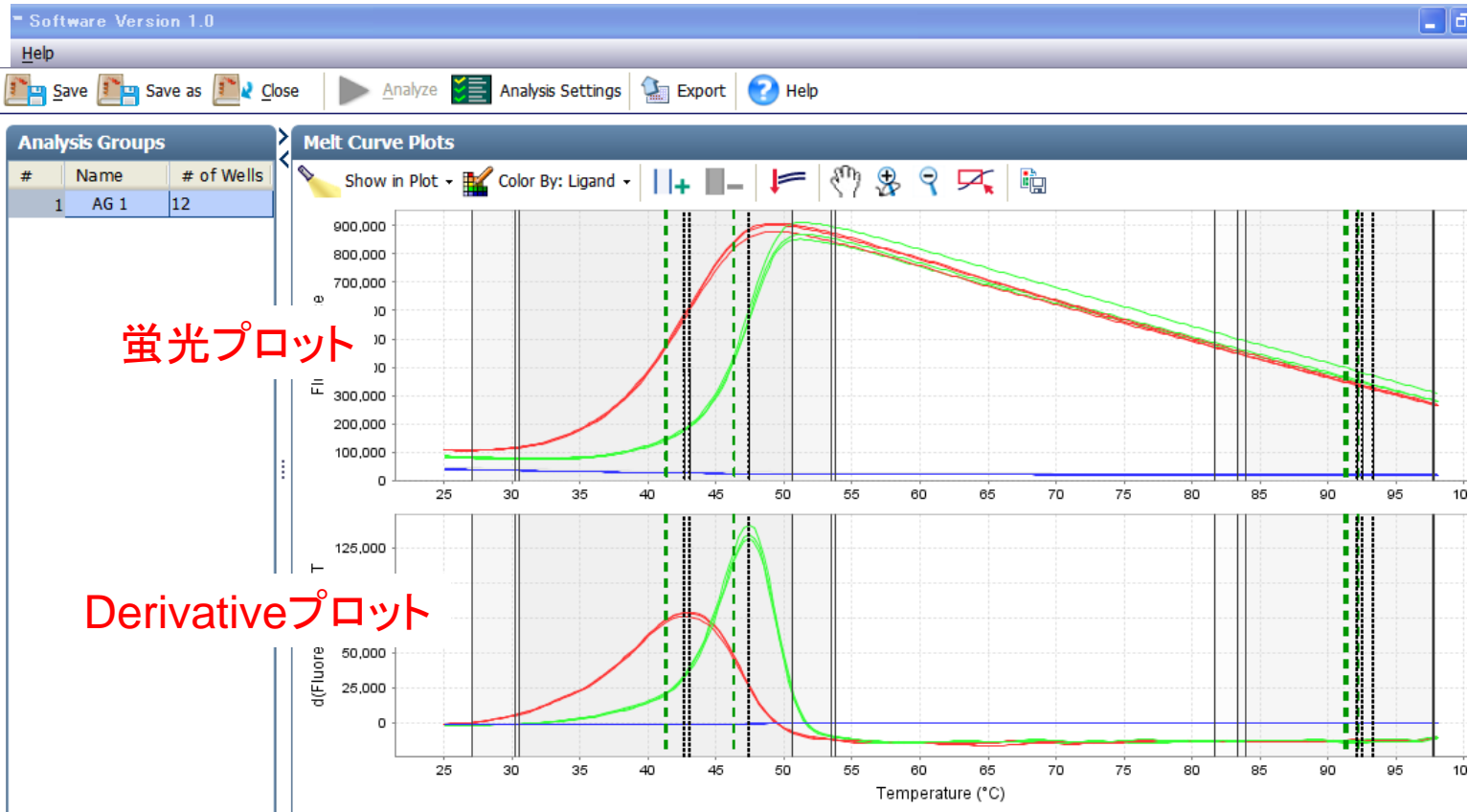
Analyze

### Plate Setup for: 110801PTS\_control.eds

Assign Auto-Fill Settings Show in Well Color by: Ligand Legend

	1	2	3	4	5
A	R Reference Ligand 0mM AG 1	R Reference Ligand 0mM AG 1	R Reference Ligand 0mM AG 1		
B	S Sample Ligand 1mM AG 1	S Sample Ligand 1mM AG 1	S Sample Ligand 1mM AG 1		
C	S Sample Ligand only AG 1	S Sample Ligand only AG 1	S Sample Ligand only AG 1		
D	N NPC AG 1	N NPC AG 1	N NPC AG 1		

# 解析結果



蛍光プロット

Derivativeプロット

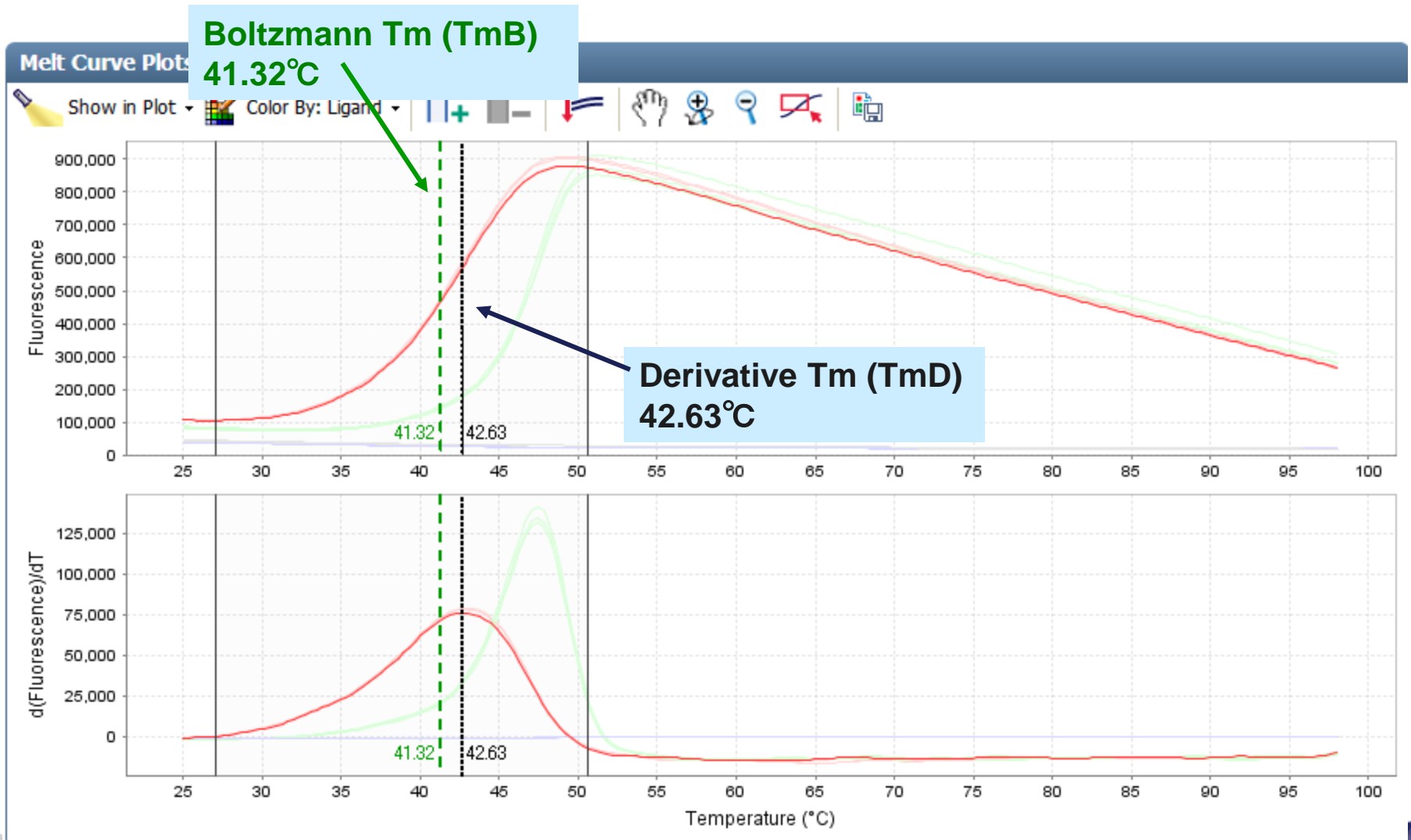
## Well Table

up by ▾

Auto Analysis Options ▾   Om

#	Well	Ligand	Task	Tm B	ΔTm B	B Fit	Tm B Start	Tm B End	⚠	Single/Multiple	Omit	Analysis Mode	Tm D	ΔTm D
1	A01	Ligand 0mM	R	41.32	-0.03	1.24	27.04	50.60	0	Single	<input type="checkbox"/>	Auto	42.63	-0.15
2	A02	Ligand 0mM	R	41.38	0.03	1.25	27.08	50.63	0	Single	<input type="checkbox"/>	Auto	43.03	0.25
3	A03	Ligand 0mM	R	41.34	-0.01	1.25	27.09	50.65	0	Single	<input type="checkbox"/>	Auto	42.68	-0.10
4	B01	Ligand 1mM	S	46.31	4.97	1.39	30.49	53.47	0	Single	<input type="checkbox"/>	Auto	47.40	4.62
5	B02	Ligand 1mM	S	46.30	4.96	1.42	30.24	53.79	0	Single	<input type="checkbox"/>	Auto	47.43	4.65
6	B03	Ligand 1mM	S	46.29	4.95	1.42	30.54	53.52	0	Single	<input type="checkbox"/>	Auto	47.45	4.67
7	C01	Ligand only	S	91.40	50.06	1.09	83.35	97.71	2	Single	<input type="checkbox"/>	Auto	92.50	49.71
8	C02	Ligand only	S	92.24	50.89	1.10	83.95	97.73	1	Single	<input type="checkbox"/>	Auto	92.16	49.37
9	C03	Ligand only	S	91.22	49.87	1.17	81.67	97.76	2	Single	<input type="checkbox"/>	Auto	93.28	50.50
10	D01		N						0	Single	<input type="checkbox"/>	Auto		

# 解析結果



# 解析結果

**Boltzmann Tm (TmB)**  
シングルピーク用

**Derivative Tm (TmD)**  
マルチプルピーク用

Well Table

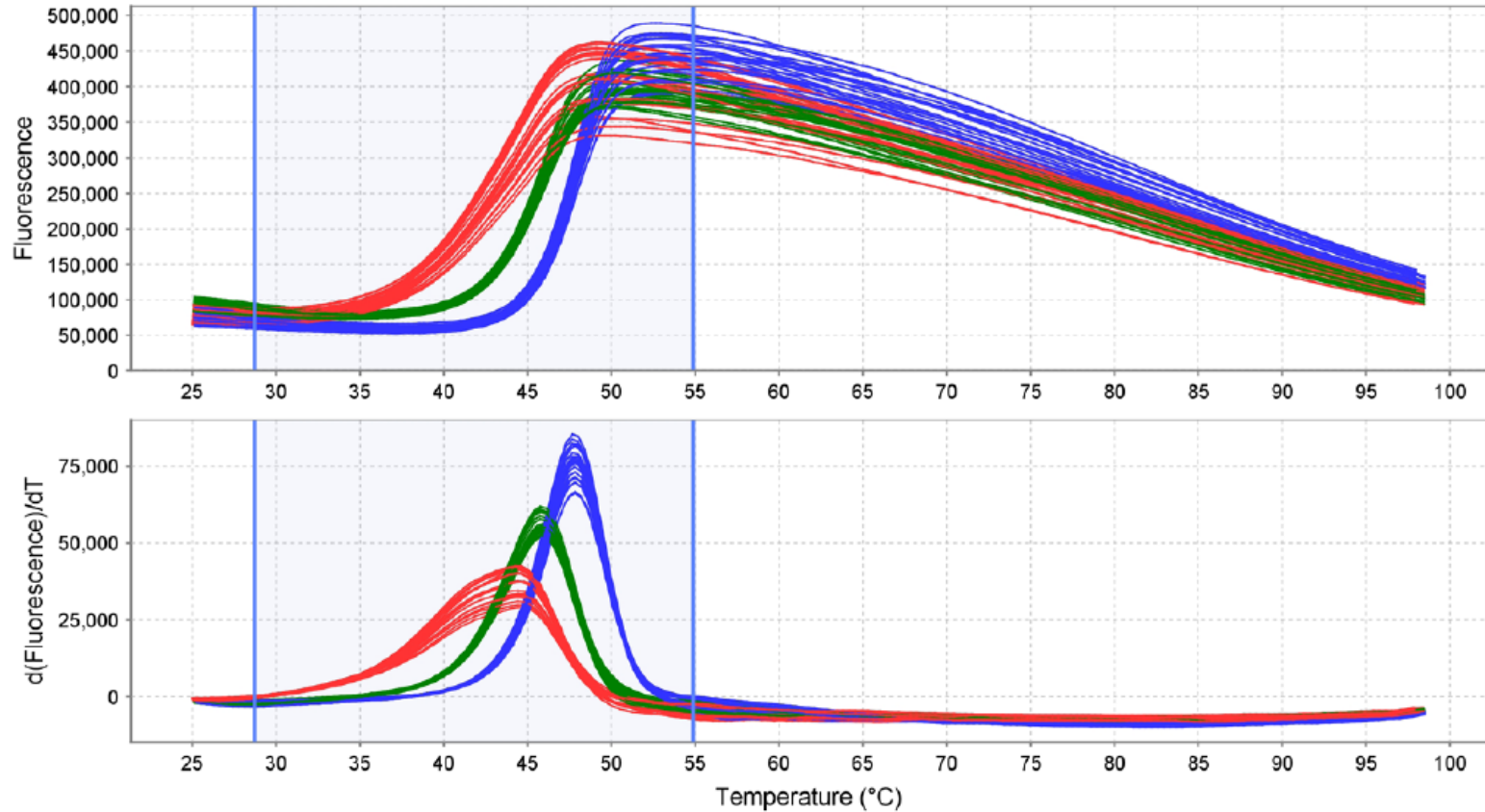
Show in Table ▾ Group by ▾ Auto Analysis Options ▾

#	Well	Ligand	Task	Tm B	ΔTm B	B Fit	Tm B Start	Tm B End	Single/Multiple	Omit	Analysis Mode	Tm D	ΔTm D
1	A01	Ligand 0mM	R	41.32	-0.03	1.24	27.04	50.60	0 Single	<input type="checkbox"/>	Auto	42.63	-0.15
2	A02	Ligand 0mM	R	41.38	0.03	1.25	27.08	50.63	0 Single	<input type="checkbox"/>	Auto	43.03	0.25
3	A03	Ligand 0mM	R	41.34	-0.01	1.25	27.09	50.65	0 Single	<input type="checkbox"/>	Auto	42.68	-0.10
4	B01	Ligand 1mM	S	46.31	4.97	1.39	30.49	53.47	0 Single	<input type="checkbox"/>	Auto	47.40	4.62
5	B02	Ligand 1mM	S	46.30	4.96	1.42	30.24	53.79	0 Single	<input type="checkbox"/>	Auto	47.43	4.65
6	B03	Ligand 1mM	S	46.29	4.95	1.42	30.54	53.52	0 Single	<input type="checkbox"/>	Auto	47.45	4.67
7	C01	Ligand only	S	91.40	50.06	1.09	83.35	97.71	2 Single	<input type="checkbox"/>	Auto	92.50	49.71
8	C02	Ligand only	S	92.24	50.89	1.10	83.95	97.73	1 Single	<input type="checkbox"/>	Auto	92.16	49.37
9	C03	Ligand only	S	91.22	49.87	1.17	81.67	97.76	2 Single	<input type="checkbox"/>	Auto	93.28	50.56
10	D01		N						0 Single	<input type="checkbox"/>	Auto		

ΔTmはReferenceサンプルとのTm差

# Protein Thermal Shift™

## タンパク質とリガンドの結合安定性測定



Legend

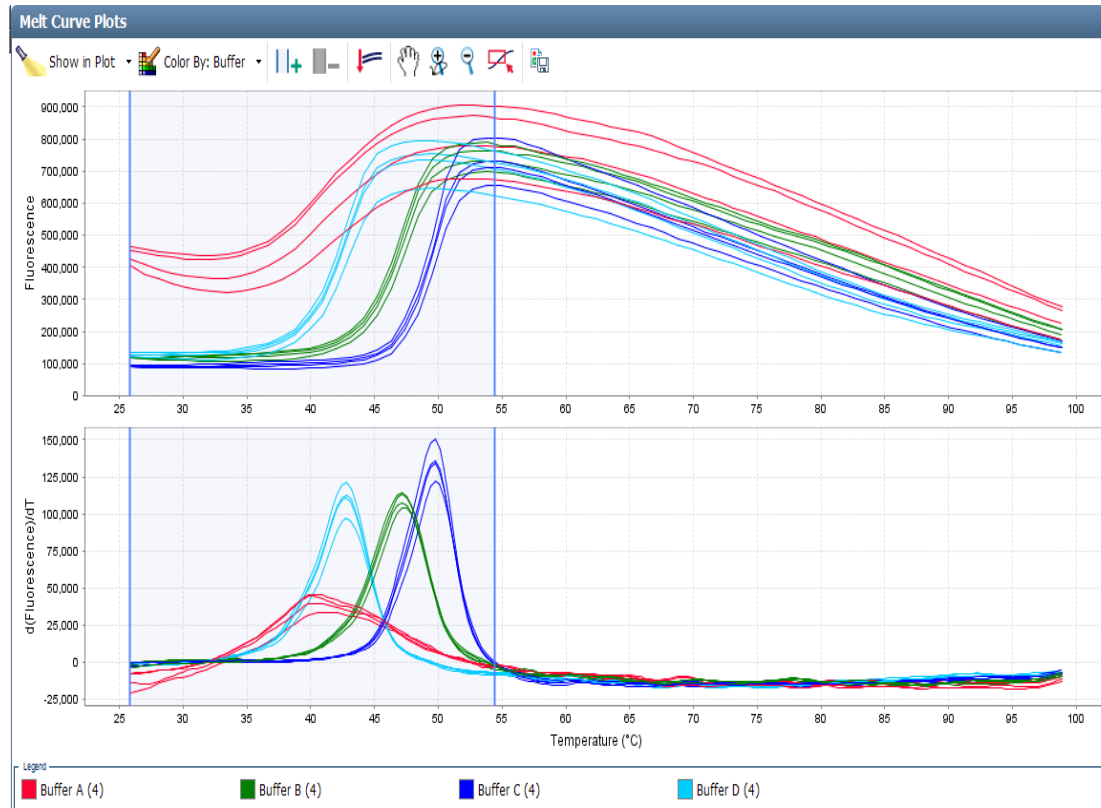
■ Ligand 0 mM (24)    ■ Ligand 0.1mM (24)    ■ Ligand 1 mM (23)

life



# Protein Thermal Shift™

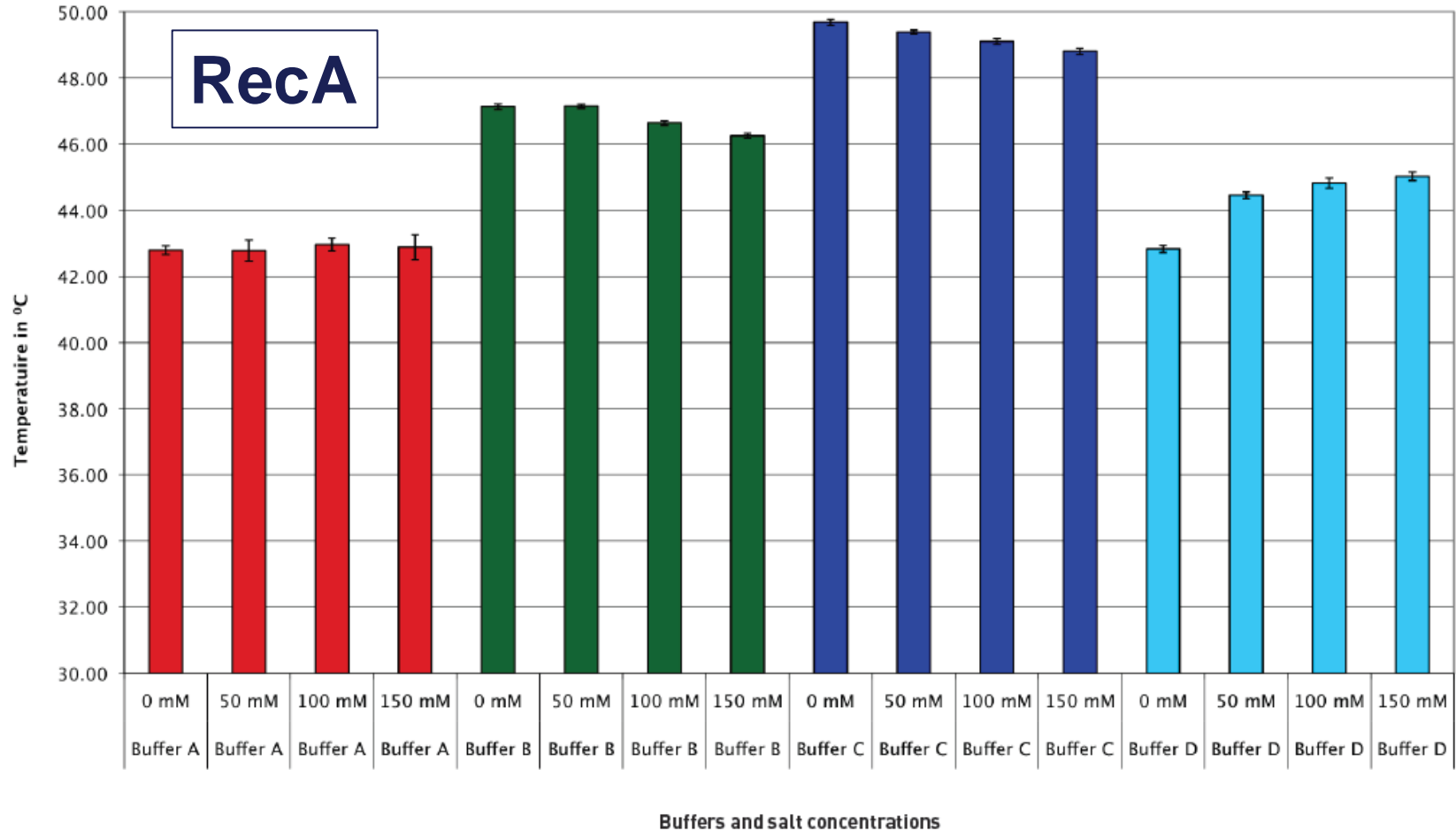
## バッファー組成の違いによる安定性の測定



Sample	Temperature	Buffer	Line
RecA in Buffer A4	38.6	Na citrate pH 5.5 + 150 mM NaCl	<span style="color: red;">■</span>
RecA in Buffer B4	45.6	KPO4 pH 6.0 + 150mM NaCl	<span style="color: green;">■</span>
RecA in Buffer C4	49.0	KPO4 pH 7.0 + 150mM NaCl	<span style="color: blue;">■</span>
RecA in Buffer D4	45.0	Hepes. pH 7.5 + 150mM NaCl	<span style="color: cyan;">■</span>

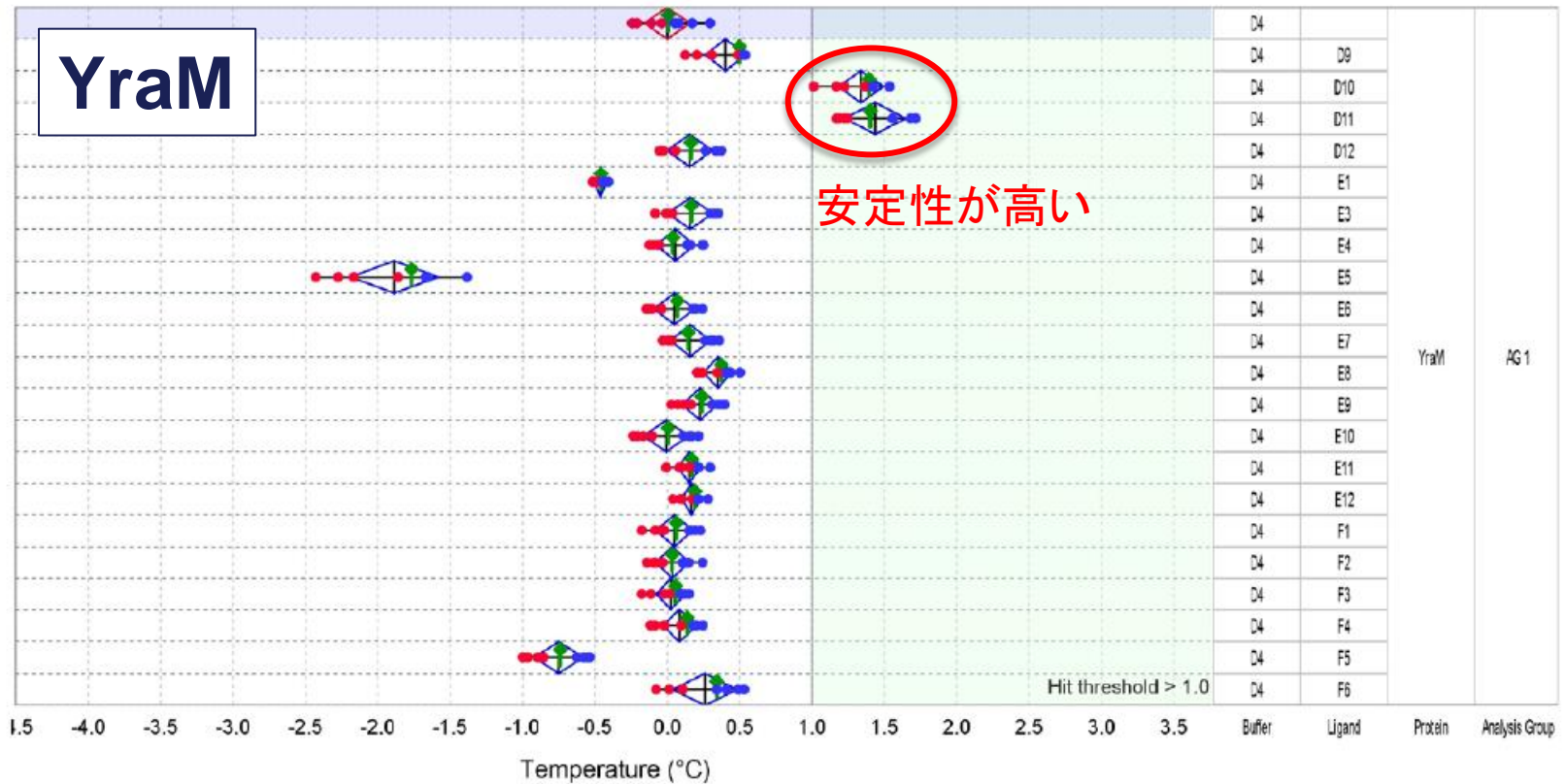
# Protein Thermal Shift™

## バッファー組成の違いによる安定性の測定



# Protein Thermal Shift™

## バッファー組成の違いによる安定性の測定



Legend

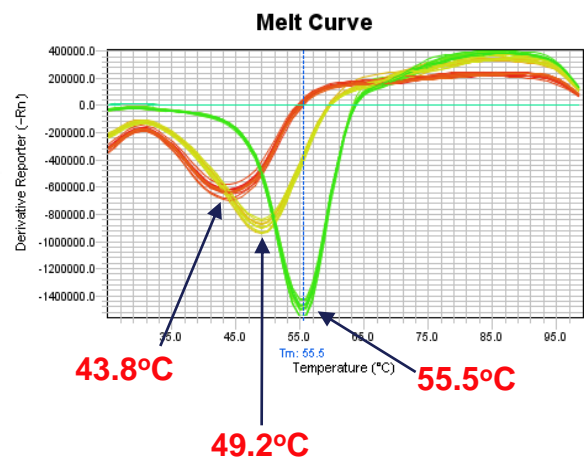
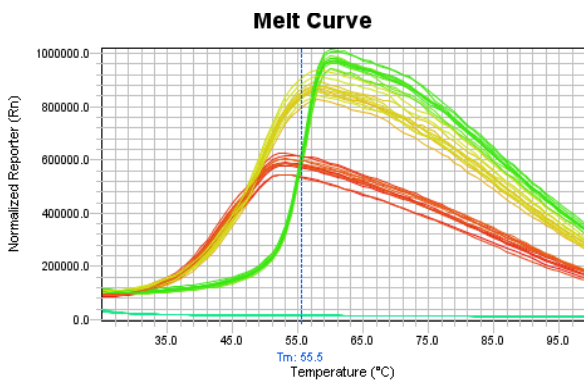
- Reference Replicate Group (1)
- Sample Replicate Group (21)
- Omitted (0)
- 20100331 Quente YraM D9through F6...
- 20091216 Quente YraM silver bullet tes...



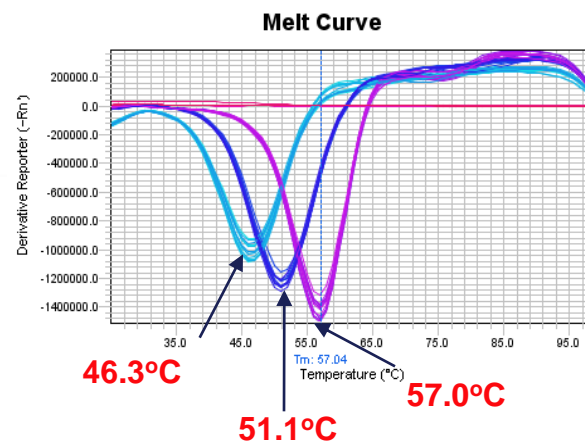
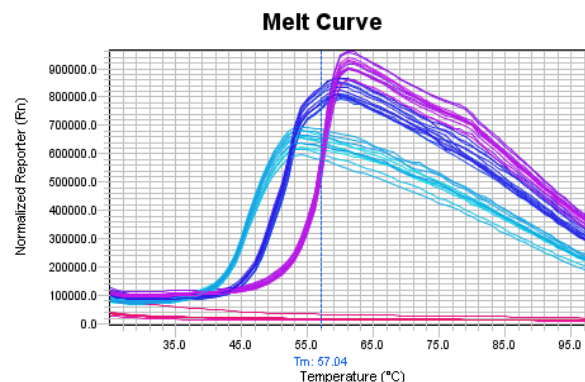
# Protein Thermal Shift™

## タンパク質の点変異における安定性の測定

Wild type, Mut1, Mut2



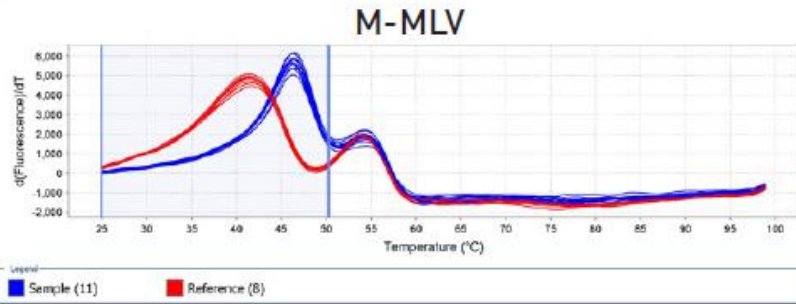
Wild type, Mut1, Mut2  
+ Ligand



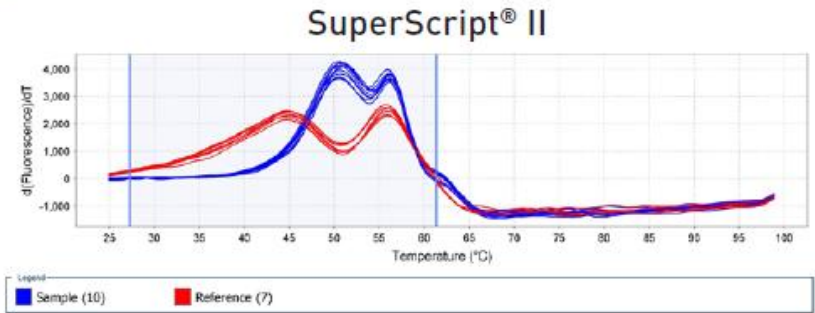
# Protein Thermal Shift™

## タンパク質の点変異における安定性の測定

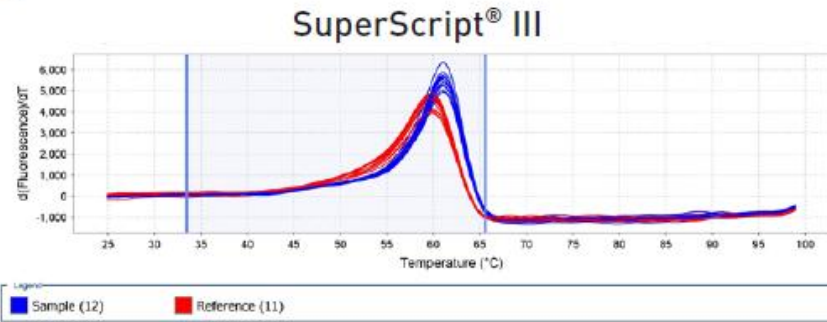
A



B



C



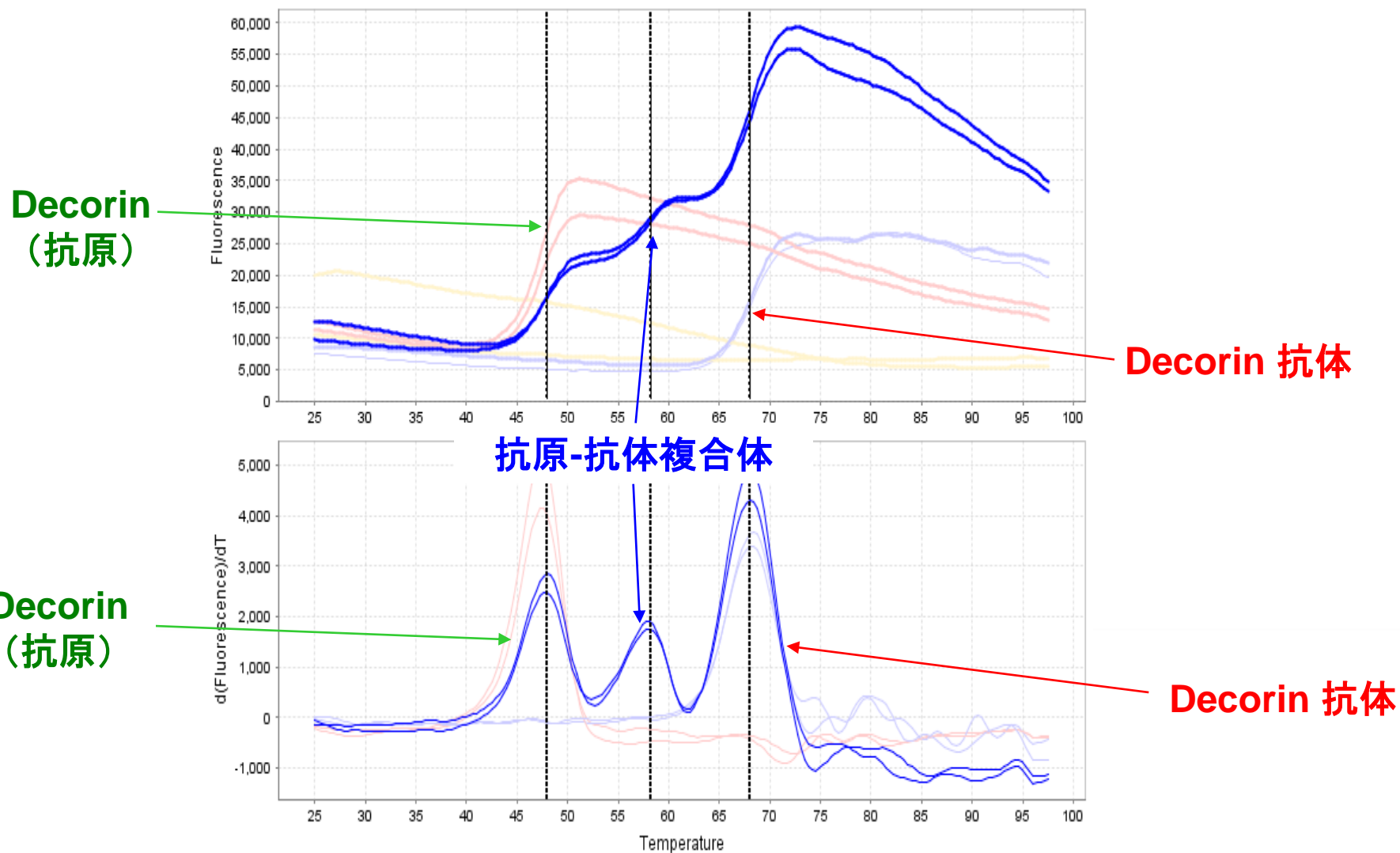
— DNAなし  
— DNAあり

[http://www3.appliedbiosystems.com/cms/groups/mcb\\_marketing/documents/generaldocuments/cms\\_095306.pdf](http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_095306.pdf)



# Protein Thermal Shift™

## 抗原と抗体結合の検出例



# Ordering Information

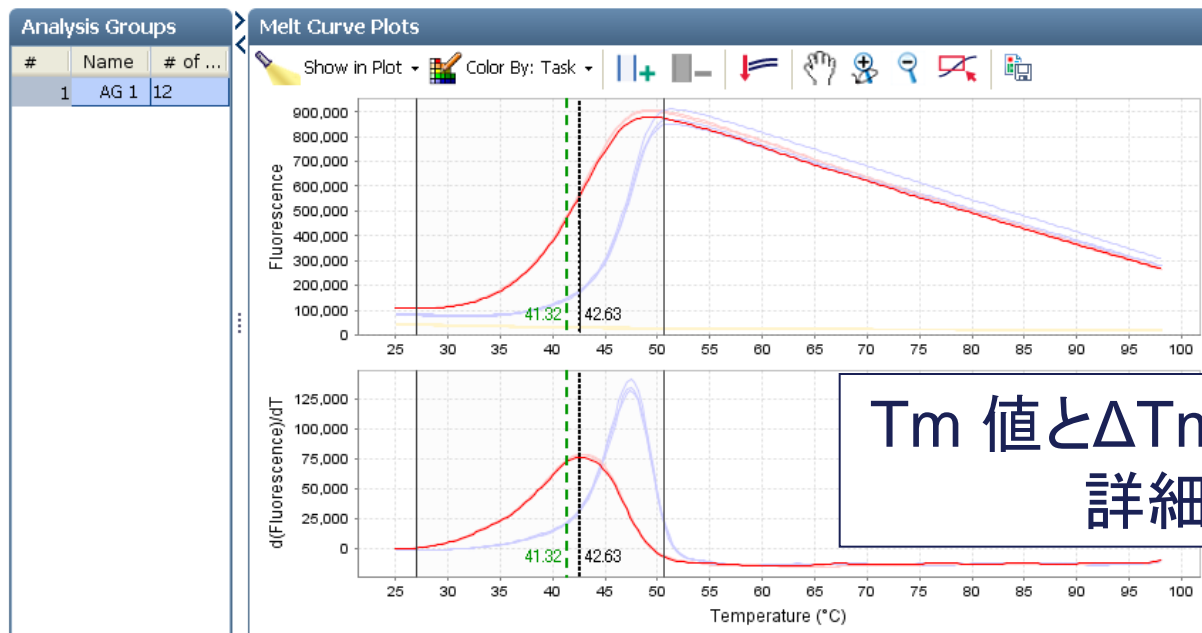
製品名	サイズ	製品番号	価格
Protein Thermal Shift™ スターターキット	2,000反応 + 100 コントロール反応	4462263	¥44,000
Protein Thermal Shift™ Dye キット	2,000反応	4461146	¥19,000
Protein Thermal Shift™ Software v1.0	10 ライセンス	4466037	¥480,000

2011年7月現在

- これから実験を開始する場合はスターターキット (4462263)がお勧め
- 継続して試薬を買い足す場合は Dye (4461146) を購入
- T<sub>m</sub> 値はある程度であればリアルタイム PCR 付属ソフトでも対応可能
- 大規模にスクリーニングを行う場合は PTS software v1.0 があると便利



# PTS software v1.0 で Tm 値を自動解析



Well Table

Show in Table  Group by

Auto Analysis Options  Omit

#	Well	Protein	Ligand	Task	Tm B	$\Delta Tm B$	B Fit	Tm B Start	Tm B End	Single/Multiple
1	A01	Protein 1		R	41.32	-0.03	1.24	27.04	50.60	0 Single
2	A02	Protein 1		R	41.38	0.03	1.25	27.08	50.63	0 Single
3	A03	Protein 1		R	41.34	-0.01	1.25	27.09	50.65	0 Single
4	B01	Protein 1	Ligand 1	S	46.31	4.97	1.39	30.49	53.47	0 Single
5	B02	Protein 1	Ligand 1	S	46.30	4.96	1.42	30.24	53.79	0 Single
6	B03	Protein 1	Ligand 1	S	46.29	4.95	1.42	30.54	53.52	0 Single

お試し版ダウンロード！

<https://products.appliedbiosystems.com/ab/en/US/adirect/ab?cmd=catNavigate2&catID=608641>



# 確認されている実験条件

- タンパク質量: 0.05-5  $\mu\text{g}/\text{well}$
- 蛍光色素量: 1X-20X
- pH: pH 2.0 – pH 9.0
- バッファー: HEPES、Tris、 $\text{KPO}_4$ 、NaCitrate、Glycine HCl

## 実験の流れのご提案

1. 1  $\mu\text{g}/\text{well}$  で目的タンパク質が PTS に適しているかどうか確認
2. 0.05-5  $\mu\text{g}/\text{well}$  の範囲でタンパク質量を振り、最適タンパク質量を検討
3. pH、バッファー、リガンド等、条件を変えスクリーニング実験

# 実験上の注意点

- 全てのタンパク質に適応できるわけではない
  - タンパク質によってはデータがうまく出ないものがある
- バッファー、タンパク質はキャリアータンパク質を含まないものを使用する
  - 市販の酵素等はキャリアータンパク質として BSA などを含んでいる場合がある
- 分注時にできるだけ気泡が入らないように気をつける
- タンパク質は変性しやすいので必ず氷上で操作する