



AB Applied Biosystems

Primer Express® Software for Real-Time PCR

Version 3.0

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Primer Express v3.0

Primers and Probe Design For Real-Time PCR



Primers/Probes Design Guideline

TaqMan Probe	Primer
Probe 與 Primer 的距離愈近愈好, PCR 產物大	小建議在 50-150 bp 為最佳
G/C % 為 30-80 %	
避免有重複序列的出現,尤其避免4	4個以上G的出現
Tm 值: 68-70 (Quantification assay)	Tm 值: 58-60℃
65-67 (Allelic Discrimination assay)	
Probe 長度:	Primer 長度:
13~25 bases (TaqMan MGB probe)	20 bases (Optimal)
13~30 bases (TaqMan probe)	
避免連續6個A的序列出現	3'端的前五個序列裡不能超過2個C+G
5'端第一個序列不能為G	
(如果選擇 FAM-dye 在 5'端第二個序列也不能為 G)	
選擇C比G多的strand當作probe ^b	
避免3'端的前4個序列裡含有3個或以上G	
(GGG-MGB-3' or GGAG-MGB-3') ^a	
避免probe的中間區域含有2個或以上的CC di-nucleotides ^a	

a: 針對 TaqMan MGB probe

b: 參數可選擇設定

Primers & Probes for Quantification

Automatically Design

進入 Primer Express 3.0 軟體

 $File \rightarrow New \rightarrow$ 選擇"TaqMan MGB Quantification 或 TaqMan Quantification" \rightarrow OK



Tools→按"Add DNA File" 😡,尋找序列存取位置,按下"Add",將序列檔案加入空白文件。亦

可在"Sequence" Tab 中使用 Copy & Paste 轉貼或直接輸入序列。

📶 Primer Express 3 (
<u>File E</u> dit <u>V</u> iew <u>T</u> ools	: <u>W</u> indow Help		
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📓 TaqMan® MGB Q	uantification # 1		- 🛛
Sequence Parameters	Primers / Probes Order		
🚮 File Name			
Length	Selection K	0 to 0 Double Stranded	
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		MAdd DNA File	
		🔟 🗀 Quantification 💿 🧊 📁 📰 📰	
		Image: NM_002217.fasta Image: NM_002217 Image: NM_002217	
To load a DNA file, click	he "Add DNA File" button. T	To enter data from the keyboard, begin typing	

* Primer Express Software 只能接受.dan, .txt, .ab1,或.abi 的檔案格式,請事先將欲分析的序列存成純文字檔即 可(若序列是從 database download 下來時,請刪除與序列無關之資料)



可勾選" Double Strand", 即會顯示 double stranded DNA sequence

M Primer Express 3.0	
<u>File Edit View T</u> ools <u>W</u> indow Help	
TaqMan® MGB Quantification # 1	- 8×
Sequence Parameters Primers / Probes Order	
File Name NM_002217.txt	
Length 2809 bp. Selection 1 to 1 🔽 Double Stranded	
TCAGCGATGG CATTIGCATG STGGCCCCTGT CTCATCITGG CTCTGCTCTC	50 🔨
AGTEGETACE GTAAACGTAE CACEGEGACA GAGTAGAACE GAGACGAGAG	100
	150
CAGCTTGGCA GCCTCTGGCT TCCCGAGAAG CCCCTTTCGG CTGCTTGGGA	200
STCSAACCST CSGAGACCSA AGSSCTCTTC GSGSAAASSC GACSAACCCT	250
	300
AACGGAGCCT CCCGGAAGGG GTGGCCAATG GCATCGAGGT CTACAGTACC	350
TTGCCTCGGA GGGCCTTCCC CACCGGTTAC CGTAGCTCCA GATGTCATGG	400
	450
AAAATCAACT CCAAGGTGAC CTCCCGTTTT GCTCACAATG TTGTCACCAT	500
ITITAGTIGA GGIICCACIG GAGGGCAAAA CGAGIGITAC AACAGIGGIA	550
	600
GRORGCUGIC ARCUGIGURG ACRUGUCAR GGROGIIICU IIIGRIGIGG	650
	700
AGTECTED SACESTTE ATCACEADET TEACETTSAC CATEGORY	750
Trangaratt (Tarrasha thataatta) hataaharta athataari	800
	850
GTTACCTACC CTGGGAATGT CAAGGAGAAG GAAGTTGCCA AGAAGCAGTA	900
To find Primers & Probes, click the "Find Primers/Probes" button	

Tools 上的選項

1. Exclude:不必要的序列可利用"Exclude Selected Bases" 將序列刪掉

- 先選取不要的序列範圍,點選 "Exclude",即可看見此區域被劃掉

TaqMan® MGB Quantification #1		- X
Sequence Parameters Primers / Probes Order		
File Name NM_002217.txt		
Length 2809 bp. Selection 1 to 50 🗹 Double Stranded		
Υ	mlm	
PEAGEGATES CATTIGEATE STERREETET CTEATETIES CTETECTEE	50	
AGTEGETACE BTAAACGTAE CACEGGGACA BAGTAGAACE GAGACGAGAG	100	
	150	
CAGCTIGGCA GCCICIGGCI ICCCGAGAAG CCCCIIICGG CIGCIIGGGA	200	
GTCGAACCGT CGGAGACCGA AGGGCTCTTC GGGGAAAGCC GACGAACCCT	250	
	300	
AACGGAGCCT CCCGGAAGGG GTGGCCCAATG GCATCGAGGT CTACAGTACC	350	
TIGCCICGGA GGGCCIICCC CACCGGTIAC CGIAGCICCA GAIGICAIGG	400	
	450	
AAAATCAACT CCAAGGTGAC CTCCCGTTTT GCTCACAATG TTGTCACCAT	500	
TTTTAGTTGA GGTTCCACTG GAGGGCAAAA CGAGTGTTAC AACAGTGGTA	550	
	600	
GAGAGCCGTC AACCGTGCAG ACACGGCCAA GGAGGTTTCC TTTGATGTGG	650	
CTCTCGGCAG IIGGCACGIC IGIGCCGGII CCICCAAAGG AAACIACACC	700	
	750	
AGCTGCCCAA GACGGCCTTC ATCACCAACT TCACCTTGAC CATCGACGGT	800	
TCGACGGGTT CTGCCGGAAG TAGTGGTTGA AGTGGAACTG GTAGCTGCCA	850	
	900	
GTTACCTACC_CTGGGAATGT_CAAGGAGAAG_GAAGTTGCCA_AGAAGCAGTA	050	



2. Junction: 如果已經知道序列上 junction 位置則可利用"Junction" J

- 框選 juction 的位置(必須含 2 bases), 再點選 "Junction", 即可看見此區域被紅色標 記起來。

TaqMan® MGB Quantification #1 Junction		J 🗙
Sequence Parameters Primers / Probes Order		
File Name NM_002217.txt		
Length 2809 bp. Selection 289 to 290 🗹 Double Stranded		
	mulu	
TCAGCGATGE CATTIECATE STEGECCTET CTCATCITES CICTECICIC	50	^
AGTEGETACE GTAAACGTAE <mark>Ca</mark> eegggaca gagtagaace gagaegagag	100	
	150	
CAGCTIGGCA GCCTCIGGCI ICCCGAGAAG CCCCTIICGG CIGCIIGGGA	200	
GTCGAACCGT CGGAGACCGA AGGGCTCTTC GGGGAAAGCC GACGAACCCT	250	
	300	
AACGGAGCCI CCCGGAAGGG GIGGCCANIG GCAICGAGGI CIACAGIACC	350	
TIGCCICGGA GGGCCIICCC CACCGGI <mark>IAC</mark> CGIAGCICCA GAIGICAIGG	400	
AAAATCAACT CCAAGGTGAC CTCCCGTTTT CCTCACAATG TTCTCACCAT	450	
	500	
	550	
GAGAGCCGT	600	
CTCTCGGCAS TTGGCACGTC TGTGCCGGTT CCTCCAAAGG AAACTACACC	700	
	750	
AGCTGCCCAA GACGGCCTTC ATCACCAACT TCACCTTGAC CATCGACGGT	800	
TCGACGGGTT CTGCCGGAAG TAGTGGTTGA AGTGGAACTG GTAGCTGCCA	850	
	900	
GTTACCTACC CTGGGAATGT CAAGGAGAAG GAAGTTGCCA AGAAGCAGTA	050	×

3. 在"Parameters" Tab 中可看到 Primer/Probe Tm 值設定及其他 Primers/Probe 設計之規範

Sequence Parameters Primers / I	Probes Order		
5			
Parameter		Value	
Primer Tm			· · · · · · · · · · · · · · · · · · ·
Min Primer Tm		58	
Max Primer Tm		60	
Max Difference in Tm of Two Pr	imers	2	
Primer GC Content			
Min Primer %GC Content		30	
Max Primer %GC Content		80	=
Max Primer 3' GC's		2	
Primer 3' End Length		5	
Primer 3' GC Clamp Residues		0	
Primer Length			
Min Primer Length		9	
Max Primer Length		40	
Optimal Primer Length		20	
Primer Composition			
Max Primer G Repeats		3	
Max Num Ambig Residues in Pri	mer	0	
Primer Secondary Structure			
Max Primer Consec Base Pair		4	
Max Primer Total Base Pair		8	
🖃 Primer Site Uniqueness			
Max % Match in Primer		75	
Max Consec Match in Primer		9	
Max 3' Consec Match in Primer		7	~



Seq	unno	e Parameters	Planars / Pho	Cent Order										
3	Can	didate Pierers I	Prober											
	1	Fwd Start	Fudien.	FedTa	Feed SGC	flev Start	Rev Len.	RevIm	Rev SGC	Probe Shart	Probe Le	Probe Tm	Probe 10C	An
	1	1343	15	50	60	13/6	19	50	50	1,753	17	70	59	82
	2	1040	19	58	58	1099	22	59	45	1060	17	68	41	79
	3	1040	19	59	58	1100	22	59	屿	1060	17	(69	41	80
	4	1040	19	58	58	1100	22	59	45	1060	18	69	39	-180
	5	1040	19	58	58	1100	22	59	45	1061	17	69	41	80
	<u>8.</u>	1822	21	58	57	1887	20	60	55	1853	14	64	(71	- 164
	7	1822	21	58	57	1887	19	58	50	1853	14	68	271	64
	9	1822	23	59	57	1889	18	59	61	1953	14	69	[2]	95
	2	1822	21	58	37	1892	19	59	63	1853	14	68	71	84
	10	2169	21	58	57	2245	20	59	55	2204	15	69	167	(84
	11	2169	21	58	32	2245	20	55	55	2206	16	69	69	- 184 - 1
	12	2169	21	58	57	2245	20	59	55	2209	15	69	73	84
	13	2169	21	58	57	2245	20	199	55	2210	14	168	79	-94
	14	2169	21	198	157	2245	20	59	55	2210	(15		(72	- 14
		1273								_				
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•	Sec	orday Structu												
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	B	evenue Primer				_								
	16	ANSCECTOC	ACTOCASA											
	6													
		CADDACTOR	ATCCCC.											

回到 "Sequence" Tab, Tools → Find Primers/Probes , 軟體即開始找尋適當的 Primers/Probe pairs

Primer Express 軟體會找到 Candidate Primers & Probe pairs, 一次 search 最多能找到 50 種組合,這 些組合列於"Primers / Probes" Tab 中。中間"Location"說明 primers & probes 在序列中的相對位置, 在横線上方的數字代表起始位置,横線下方則代表終止位置。

"Sequence" Tab 中會顯示出與"Primers / Probes" Tab 相對應的一對 Primers/Probe: 粉紅色片段是 Probe 的位置,藍色代表 Forward Primer,黄色片段則為 Reverse Primer,如下圖所示。(*但並不表 示此為最佳的設計)

📓 TaqMan 🖲	MGB Qua	ntification	≇1							
Sequence	Parameters F	rimers / Probe	es Order							
File Nam	e NM_0022	17.txt								
 Length	2809 bp.	Selection	289	to	289	✓ Double Strand	led .			
turning turnet	mmm	սսսսևս	uuuuluu	սոսը	պապե		սողու	uuduuuu	mhnnin	
CATCGGGGGC	AAGTTCCCCT	TOTOTOTOT	GGGCTTTGGC	00000T01	1 64					-
GTAGCCCCCG	TTCAAGGGGA	ACATATTGGA	DODITIO00	UDATTDTT	. Vn 1/"T					
ATTATAACTT	CCTGGAGAAC	ATGGCCCTGG	AGAACCATGG	GTTTGCCC	GG					
TAATATTGAA	GGACCTCTTG	TACCGGGACC	TCTTGGTACC	CAAACGGG	scc					
CGCATTTATG	AGGACTCTGA	TGCCGA <mark>TTTG</mark>	CAGTTGCAGG	GCTTCTAT	GA					
GCGTAAATAC	TCCTGAGACT	ACGGCT <mark>AAAC</mark>	GTCAACGTCC	CGAAGAT/	CT					
aasaataaaa		TALAAAATAT	0010170010	T.400000						
GGAGGIGGCC	TTCCCACIGC	10ACGGGIGI	CONCAUNTOURU	ATCCCC67	NGA Mar					
CUTCCACCOO	1100010AC0	ACTOCCCACA	CUTUIACUIC	A1000000	.CI					
ACGCTATCCT	GGACCTCACC	CAGAACACTT	ACCAGCACTT	CTACGATO	GC					
TGCGATAGGA	CCTGGAGTGG	GTCTTGTGAA	TGGTCGTGAA	GATGCTAC	CG					
TCTGAGATCG	TGGTGGCCGG	GCGCCTGGTG	GACGAGGACA	TGAACAGO	TT					
AGACTCTAGC	ACCACCGGCC	CGCGGACCAC	CTGCTCCTGT	ACTTGTCO	5AA					

Primer Express Operation Guide

在 "**Primers / Probes**" Tab中會將每對Primers/Probe的組合列出來,請從中挑選出適當的組合,挑選 方式可依據第 2 頁primer/ probe design guideline。在MGB Probe的篩選中,亦可在"**Parameters**" Tab 中加選<u>"C"比"G"多的序列</u>作為進一步篩選的參數。

Sequence Parameters Primers / Probes Order	
	0.1
Parameter Max Rimer & Reneats	Value
Max Hime of Hepedas	0
Max Ham Annoig Hestades in Hinner	0
Max Primer Concern Base Pair	4
Max Filmer Consect Dase Fair Max Filmer Total Dase Fair	8
	0
Max 2 Match in Primer	75
Max & Machini Timer	9
Max 21 Casses Matchini Initia	7
	<i>r</i>
	C0
Marrielen Te	70
	70
Min Braha 200 Content	20
Mini Flobe & dC Content	00
	80
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Max Probe Secondary Studente	4
Max Flobe Consec Dase Fail	4
	0
	0
Mint Amplified region i m	05
Max Amplined negron mi	60 E0
Min Amplinea negion Lengm	150
Max Amplinea Region Length	150
U deneral	FO
Max Frimers / Frodes	100

決定 primer/ probe set 之後則可進行存檔,存檔的方式從 File → Save As 存檔。如果想將所選擇的 primer/ probe set 單獨儲存,可利用 Export → Order Info...的方式,或者要儲存 50 個 Primers/Probes 清單,可點選 Export → Primers/Probes List...,以上兩種方式都可存成.txt 檔案,在 Excel 中開啟。

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	xport		Seq.	vence	Feed 200C	Rev Stat	Rev Len	Rev Tm	Rev 2GC	Probe Start	Probe Le	Probe Tm	Probe 200C	Αα	
	age S	etup., Ctrl+	U Para	meters	60	1395	19	59	53	1359	17	70	59	82 ^	
	144	CM	p Prim	er/Probe List	58	1099	22	59	45	1060	17	68	41	79	
-			Orde	e leto	58	1100	Z2	59	45	1060	17	68	41	80	
_	. 88		10	100	08	1100	22	59	45	1060	18	69	39	80	
	2	1040	21	50 . 0	57	1007	20	57	42	1051	14	69	21	00	
	7	1822	21	50	57	1997	19	59	50	1953	14	69	71	04	
	8	1822	21	58	57	1888	18	59	61	1853	14	68	71	85	
	9	1822	21	58	57	1892	19	59	63	1953	14	68	71	84	
	10	2169	21	58	57	2245	20	59	55	2204	15	69	67	84	
	11	2169	21	58	57	2245	20	59	55	2206	16	69	69	84	
	12	2169	21	58	57	2245	20	59	55	2209	15	69	73	84	
	13	2169	21	58	57	2245	20	59	55	2210	14	68	79	84	
	14	2169	21	58	57	2245	20	59	55	2210	15	70	73	84 🚩	
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			Oligo			Length		Hairpin Se	# Dimers Cross	Dimers					
	0	Forward P	rimer			21		Most Stable Structure Found Heirsies ont found							
	0	Reverse Pr	iner			20									
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	8 142 9 142 10 216 11 216 12 202 13 216 14 216 C 9 Location Prob Forwar Forwar CTTCC Revenue	TTCCAGGTO	GAGGTGAC	CANC .											
	R	everce Prime	1												
	Secondary Stuct Forward P Reverse Pr Probe Forward Primer CTTCCAGG10 Reverse Prime TGTCCAGC2		GCTGAAAG	TG											
	Seconday Stuc Forward P Pobe Forward Pime Forward Pime CTTCCAGOT Reverse Pime TGTCCAGCO Ptobe														
		ACAGGGCCC	TGCCG												

Manually Design

當軟體無法自動尋找到 Primer/Probe 組合時,先確定 Primer/Probe 想要置放的位置 開啟一個新的 Quantification Document,並把欲設計的序列檔案加入 (參照第3頁)。

1. Probe 的設計:

在"Sequence" Tab 中將 Probe 之預定序列 highlight 起來 (至少 25 bases 之長度,長度依序列結構而 定)後,先利用 Edit 中 Copy (Ctrl+C) 之功能複製序列,再依下圖至 Tools→ Primer Probe Test Tool 中,選擇欲設計的 document type (即"TaqMan MGB Quantification 或 TaqMan Quantification"),並確 認 Parameter 設定為"Default",再利用 Paste (Ctrl+V) 將序列貼在 Probe 1 欄位,從右邊即可觀察測 試序列之 Tm,%GC 和長度是否合適。

註: probe 第一個序列不能為G,且序列裡面C的數目要比G 還要多

TagMan# MOD Quantification # 2		E (#
Sequence Parameters Primers / Probes Order		
The Name NM_000217.td		
Length 2909 bp. Selection 161 to 19	5 m Frimer Fride Test Tool	8
	Parameters	
DEMODIFIED CATTROCKED CHOROCOLE CECNECTED CECTORCECCE	Document Type: Tackant Müß Guantification Tarantee: Default	Browne
ADDRESS STRANDER COLONDRA GALLANCE ANALONAS	Primers and Probit addants Quantification	
CARCITERICA OCCUCTORICI ICCCUARAASI CCCCTITICOSI CTOCTIONIA	TedMan# MGB Aleic Discrimination	
TECOMACOTE COSMISACOSA ABOOCECTEC ODDSAMAGOC GACOMACOCE	Feed Port Adelic Discrimination	Tm 3/GC Length
		0.0 0 0
AACOSAGCT CCOBSAAGOD HEGGCCAATG GCATCBAGUT CTACAETACC	Rev Pizzer	The NGC Length
TRACTORIA BRECTTOCC CACORDITAC OUTABCTCCA GAIGTCATES		To NOT Land
CANNARD ANNAL AND PRESENT AND AN - WEARES	Probe 1 CCAAGGTGACCTCCCGTTTTGCTCA	an sou Lenge
WATCHACT PCARGETORC CEOCOSTIES OCTOCOMES TEOLOGICAL		Ten ATT Learth
TITTATITON OUTFCCACTO GAUGOCAAAA COMITUTIAC AACADIGUTA	Picke 2	0.0 0 0
	1.00	(00.17.17.17.17
MENOCOLC MCCOLOCA ACADOCCA ODADILLCC 1110410100		
recommendation and an and a second second second second	Secondary Structure	
TROODER CATCORD TOACCAST CATCORDAY AND ANOTHER	and the second second second	
DOGACOBULT CTOCCOGAAG TAUDRITUM AUTOGAACTO UTAOCTOCCA	Uigo Length	and a second
	🕑 Forward Primer 0	
STRACTRCC CTOSGAADUT CAAGGAGAAG GAAGTTGCCA AGAAGCAGTA	O Reverse Primer 0	
CAATOSATOS GAOCCITACA OTTOCECTEC CEECAAOBOT ECETOSECAE	O Probe 1 25	
	Profee 2 0	
такимааст ататесская асмалковс свосттоятс мавостств	These Demondeer Thickney	
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services and the service and and another the		1300

此時若 Tm 超過設定值(68℃ to 70℃),可直接在 Probe 1 欄位中框選不同的序列片段,並觀察右邊 對應的 Tm,%GC 和長度,以找出最適當的 Probe (請參照 primer/ probe design guideline),若框選到 適合的片段,點一下"Trim",就可以將未框選的序列直接刪除,而留下需要的 Probe 序列。

🛅 Primer Probe Test	Tool	×
Parameters		
Document Type: Taqt	Man® MGB Quantification 💌 Parameter: Default 💌 Browse	
∟ Primers and Probes—		
	Tm 2/60 Length	
Fwd Primer		
Bey Primer	Tm %GC Length	
The very miller		
Probe 1	CCAAGGTGACCTCCCGTTTTGCTCA 70.0 61 18	
Probe 2	Tm %GC Length	
Flobe 2	0.0 0 0	
Trim		



當 Probe 序列確定之後,回到"Sequence" Tab 中將 Probe 之確定序列 highlight 起來並利用 Edit →

🇋 😂 📙	🎐 🕅	🗅 🗋 🗙	🔳 🕨	<u>A</u> 11	→	• الله الله الله الله	re 🕜 🛒 🗛
📓 TaqMant	® MGB Qua	ntification	≇1			Probe	
Sequence	Parameters F	^p rimers / Probe	es Order				
🔐 File Nar	ne NM_0022	217.txt	I				
Length	2809 bp.	Selection	161	to	178	Double Stranded	
L						<u></u>	
CAGCTTGGCA	GCCTCTGGCT	TCCCGAGAAG	CCCCTTTCGG	CTGCTTGGG/	۱.		
GTCGAACCGT	CGGAGACCGA	AGGGCTCTTC	GGGGAAAGCC	GACGAACCCT	[
AACGGAGCCT	CCCGGAAGGG	GTGGCCAATG	GCATCGAGGT	CTACAGTAC	:		
TTGCCTCGGA	GGGCCTTCCC	CACCGGTTAC	CGTAGCTCCA	GATGTCATG	3		
AAAATCAACT	CCAAGGTGAC	CTCCCGTT	GCTCACAATG	TTGTCACCAI	[
TTTTAGTTGA	GGTTCCACTG	GAGGGCAAAA	CGAGTGTTAC	AACAGTGGTA	1		
GAGAGCCGTC	AACCGTGCAG	ACACGGCCAA	GGAGGTTTCC	TTTGATGTG	5		
CTCTCGGCAG	TTGGCACGTC	TGTGCCGGTT	CCTCCAAAGG	AAACTACACO	:		
AGCTGCCCAA	GACGGCCTTC	ATCACCAACT	TCACCTTGAC	CATCGACGGI	Γ		
TCGACGGGTT	CTGCCGGAAG	TAGTGGTTGA	AGTGGAACTG	GTAGCTGCC/	۱.		

之後,可利用 Tools → Find Primers/Probes ▶,找出與設定之 Probe 配對的 Forward / Reverse

Primers。若自動搜尋無法找到配對的 Primers 可依以下步驟進行 **Forward / Reverse Primers** 的設計。



2. Forward Primer 設計:

在"Sequence" Tab 中將預定 Primer 序列 highlight 起來(至少 25 bases 之長度,切勿與 Probe 序列重 疊)後,先利用 Edit 中 Copy (Ctrl+C)之功能複製序列,再依下圖至 Tools → Primer Probe Test Tool 中,利用 Paste (Ctrl+V) 將序列貼在 Fwd Primer 欄位,從右邊即可觀察測試序列之 Tm,%GC 和長度是否合適。

Seguence Parameters Primers / Probes Order	
File Name NM_002217.txt	
Length 2909 bp. Selection 125 to Internet Probe Test Tool	X
Parameters	
CARCTEGORA GOLICEGORE TOCOMONAS COCCETTORS CIOCITIONS	
оторилская совидеская марастетте воздалларсе сакодалесст обсилени туре. Тадиани нов сидинисаной 🔍 наказые Совран	BIONDE
Primers and Probes	
AACEGUAGCET COCEGUARGES GTEGOCEARTS GCATCEARGET CTACASTACC	
TIGOCTOBIA DESCRIPTION CALIFICATION CONTRACTOR DATACTOR	Tm 2GC Length
had and been added to be and	57.9 48 23
AAAATCAACT COAAGSTGAC CECCONSTITT GETCACOATS TEGECACCAT	Tm %GC Length
TTETAGETGA OSTICCACTS GASSOCAAAA CSASTSTIAC AACAGEGGEA	0.0 0 0
Probe 1 CCAAGGTGACCTCCCGTT	Tm 20C Length
SAGAGEGITE AACCOTIGEAG ACACOGECAA GSAGUITTEE TITGATOTO	70.0 61 18
CTCTC080A8 TT98CA03TC T0T90C003TT OCTOCAAA88 AAACTACACC Probe 2	Tm 26C Length
	0000
ACTOCCAA GACODOCTTC ATCACCAACT TCACCTTUAC CATOBACOST	
TOSKOSSUTT CTUCCUSANG TAETOSITUA ASTOLAACTO GIAOCTOCCA	
Secondary Structure	
Office Creative Creative Contractive Contractive Office Length Flager Set Direct	Cross Dimers
Controlation december of the first of t	
ACTIVITY OF CONTRACT OF THE ACTIVITY OF THE AC	
OCTOTITION OTICITIONS TUTOMARC AUTOCAODS ACTICOTION Show Secondary Structure	
AAASTCACCT TOSAGCTAAC CTACGAGGAG CTRCTGAAGA GECACAAGG	1000
TTECHT00A AGETOSATES GAUGETCETE GACGACITET CONTRITCE	1400
	1430

確認 Forward Primer 的 Tm 值在 58-60℃,如果 Tm 值不符合,可直接在 Fwd Primer 中框選不同的 序列片段,並觀察右邊對應的 Tm,%GC 和長度,以找出最適當的 Primer (請參照 primer/ probe design guideline),若框選到適合的片段,點一下"Trim",就可以將未框選的序列直接刪除,只留下需要 的序列。

當 Fwd Primer 序列確定之後,回到"Sequence" Tab 中將確定序列 highlight 起來,再利用 "Forward Primer" →固定起來,此時的 Fwd Primer 位置會被標定成藍色。

TagMan® MGB Quantification # 1	
Sequence Parameters Primers / Probes Order	
File Name NM_002217.64	
Length 2809 bp. Selection 126 to 148 🕑 Double Stranded	
Granden and Antonia Antonia Antonia and Antonia	
Calcinger account acco	200 🔷
GEOMACCIE COMBACCIA ADDICICITE ODDIAANDEE DACIAACCEE	250
	300
ACCORDANCE CONSIGNATE OCEODERE CENTRE	350
Tracercosa associatico: excessitate caracterea galarearos	400
	450
ANALOW COMPLEX COOLINE COOLINE RECOOL	500
TITIABITGA OUTCOACTO GADDOCAAAA COASTOTIAC AACASTOTIA	550
	600
GARAGODIC ANODIDONI ACADISCAN GARGITIC TITUATOTIS	650
CECTOBORAS ITORCADITE DETOCODITE CETECAAADS AAACEACACE	700
	750
ABCTOCCAR GROBECTIC ALCRCART TCACTURE CATORODI	800
TCBACBBUTT CTGCCBBAAG IAGTBUTTGA AGTBBAACTG GIAGCTBCCA	850
	900
OTTACCIACC CIODGADIGI CAAGGAGAAG GAAGITGCCA AGAAGCAGIA	950
CARTOGATOG GACCETTACA OFFICETETE CTECANOGOT ICITOSTCAT	1000
	1050
TSAAASOCT STSTCCCASS SCAASACOSC COSCTINETC AASOCCTCTS	1100
ACTITECOM CACAGGIECE OSTECEGOS GEOSMACIAS TECOSMANC	1150
	1200
OSAGGAAGTT GAAGAAGTTC ACAGTCTCGG TCAACGTGGC TGCAGGCAGC	1250
OCTOCITICAA OCTOCITICAAS INTEGASAGOC ASTITUCADOS ACSILOCUTOS	1300
	1350
AMAGTOROCT TOSAGCIAAC CIACGAGGAG CIGCIGAAGA GOORCAAGGG	1400
TITCHETORE AND CONTRACTOR CANCELET CONTRACTOR	1450 🚽
1	



3. Reverse Primer 設計:

在"**Sequence**" Tab 中將預定的 Reverse 序列 highlight 起來(至少 25 bases 之長度, 切勿與 Probe 序列 重疊)後, 然後利用 Edit →" Copy Complement "將序列貼到 Tools → Primer Probe Test Tool 中的 Rev Primer 欄位。

Sequence Par	ameters Primer	rs / Prober	: Order								
🛐 File Name	NM_002217.6	đ			🔛 Primer Probe Test	Tool					
Length	2809 bp. 5	election	229	to 25	Parameters						
Y			mulm	uuuluuu	Document Type: Tak	Man® MGB Quantification	Parameter.	Default	× [Втонняе	
CARCITORICA OF	CTCIONT TOO	COAGAAG (COCCTTROSS	CTOCTTODGA	Primers and Probes						
STOSAACOST CO	DOAGACCISA ADD	SCECTEC (0000444000	GAOSAACCCT				Te	(sec	Levela	
					Feed Primer	CAATGGCATCGAGGTCTA	CAGTA	57.9	400	21	
AAOBGAGOCT CO	COSAA000 0T0	9C <mark>CAAT</mark> G	BCATCSA60T	CTACAUTACC				In	280	Levolh	
TIGCCIOSGA GO	ROCTTOCC CRO	00 <mark>211AC</mark>	OUTAOCTOCA	GATIGTCAT 00	Rev Primer	TCCACATCAAAGGAAACCT	TCCTT	59.8	43	23	
								Tm	260	Length	
ANATOMCI C	ANDOING CIC	0001111 0	OCICACAAI0	TIUICACCAI	Probe 1	CCAAGGTGACCTCCCGTT	*	70.0	61	18	
IIIIA0IIIA G	ALECCACED GAO		COMBTOTTAC	VICOD1001N				Tm	%6C	Length	
CHORDCOSTC AN	CONTROLMS ACAD	1222284	CONTENSION	TTRATITOR	Probe 2			0.0	0	0	
CTCTC00CAG T	DOCACIEC TIETO	ACCOUNTS (OCTOCAAAOC	AAACTACACC	Trim						
ACCOUNT OF	CONCETTE ATC	ACCANCT 1	TCACCTEGAC	CATCGACGET	Secondary Structure	e					
TOSACODETT CI	DOCCOGAAS TAST	I ADTTEN	AUTOGAACTO	GTABCTOCCA	Oligo	Length	Haipin	Self Dimers Cross Dimers			
STRACTACC C	DODDAATIST CAAC	2440465	ALCONTRACA	AGAAGCASTA	Forward Primer	23					
CAATOGATOG GA	COCTTACA OTTO	OCTOTIC (CTECAAORT	TCTTOTCAT	O Reverse Primer	23					
					O Probe 1	18					
TOAAAAOOCT GT	INTECCASE SCA	NGACODC (соострояте	AAGGCCTCTG	O Probe 2	0					
ACTITICOGA CA	CARGOTOC OFT	ICTIGCOS (BOOGAACCAG	TTOOBGAGAC			_				
					Show	Secondary Structure					
GGAGGAAGTT G	RAGAAGTEC ACAG	STCTORS 7	TCAACOT00C	TOCAGOCAGO	U					_	250
OCTOCTIONA O	TCTTCAAS ISIO	CASAGOC /	ASTTOCACCS	NOTICOTICS						1	1300
										1	1350
AAASTCACCT TO	SUBJECTARC CTA	COADDAG (CTOCTOAAGA	00CACAA000						1	1400
TTECAGEOGA AC	SCECGATES GAI	SCROCEC (GACGACTTCT	COMPATINCC						1	1450

確認 Reverse Primer 的 Tm 值也能符合在 58-60℃,如果 Tm 值不符合,可直接在 Rev Primer 中框 選不同的序列片段,並觀察右邊對應的 Tm,%GC 和長度,以找出最適當的 Primer (請參照 primer/ probe design guideline),若框選到適合的片段,點一下"Trim",就可以將未框選的序列直接刪除, 只留下需要的序列。

當 Rev Primer 序列確定之後,回到"Sequence" Tab 中將確定序列 highlight 起來,再利用 "Reverse Primer" ← 固定起來,此時的 Rev Primer 位置會被標定成粉色。

TaqMan@MGB Quantification #1 Reverse Prime Image: Content of the Cont
equence Parameters Pieners / Piotes Order Pieners / Pieners / Piotes Order Pieners / Piotes / Piote
Length 2809 bp. Selection 229 to 251 ☑ Double Standed NOTTROCK OCCUTTORS CONCUTTORS CONCUTTORS 200 2 </th
Y Y
ARCTROCK GOCTCTORET TOCOMBAND COCCTITIONS CRUCTIONS 200 200 TOMACOT COMBACOGA ABBRICICTIC GORDANARCC GACIAACCCT 250 ACOMARCCT COCEMANDE GIOCCEANT TANANCC GACIAACCCT 300 ACOMARCCT COCEMANDE GIOCCEANT TANANCCA 350
TOTAMOOT COMMANDON ABBORTETTIC GOODMANDOC GACAMADOCT 250 ANDRAND COLOMADO GTORC <mark>SATO DATCADOT HAVATA</mark> OC 350 350
ACOMARCE CCORMADD STORC <mark>SATO DATCADOT HANATA</mark> CE 300
ACOBAGET CCEREMAND STORE AND TRANSFACE 350
10021094 0000211202 CACOUNTA 20140102100 400 400
450
AMPOACE COMBINE CECCENTER OFFICEARE TREPARCY 500
TTMHTGA GUTCOCCG GADDODAAA CONFIDITAC ARONTBOTA 550
600
Transiti Theorem Antonia Constitution of the second s
bringstar skonsette aleksent execution (200
CAACADATT CTOCCOLAAS IASTORITA ASTORIACIO STACTOCCA
000
TROCTACC CTORDATET CARDARDAR GARTTROCA ASUGCARTA 050
AATOGATOS GACOCITACA GITOCRETIC CITICAAOSSI TETIOSICAT
1050
SAAABOCT GTUTCCCABD GCAGACODC CODCTTOUTC AABOCCTCTS 1100
CTITECCAR CACADDATCC CETTCIDECE DECEMANCAE TECCERARCE 1150
1200
SAGGAAUTT GARDAUTTIC ACAUTETODE TCAROTERE TECHNOLOGIC 1250
ETOCTICAA OCTUTICAAS TRICASAGOC ASTROCACOS ASTROCACOS 1300
1350
AAGTCACCT TOSAGCTAAC CTACGAGGAS CTGCTGAAGA GGCACAAGGG 1400
TICANTORA ARCTORATIO GATOCTECTE GAOSACTICE CONTRIENCE 1450



決定 primer/ probe set 後可利用 Copy & Paste 功能轉貼到一個新的 text 檔,並 save 起來做為未來參 考資料。另外,也可以把此次設計 document 進行存檔,存檔的方式從 File → Save As 存檔。

M Primer Express 3.0	
<u>File</u> Edit <u>V</u> iew <u>T</u> ools <u>W</u> indow Help	
New Ctrl+N 🐚 🛱 🗙 📕 🕨 📓 🖶 🔶 💷 🗐 📣 🔤 🖉 🛒 🚜	
ppen Ctrl+0tification ≠ 1	
Close Ctrl+L	
Save Ctrl+S	
Sav <u>e</u> As Ctrl+E 17.txt	
Export Selection 251 to 251 V Double Stranded	
Page Setup Ctrl+U	
Print Ctrl+P	
Exit GIGGCCCTGT CTCATCTIGG CTCTGCTCTC	50 🔼
AGTCGCTACC GTAAACGTAC CACCGGGACA GAGTAGAACC GAGACGAGAG	100 📃
	150 💻
CAGCIIGGCA GCCICIGGCI ICCCGAGARAG CCCCIIICGG CIGCIIGGGA	200
GICGARCCGI CGGAGACCGA AGGGCICIIC GGGGARAGCC GACGARCCCI	250
becaseart creaseeas atas terrationat createreas treaser	300
	350
	400
AAAATCAACT CCAAGGTGAC CTCCCGTTTT GCTCACAATG TTGTCACCAT	450
TTTTAGTTGA GGTTCCACTG GAGGGCAAAA CGAGTGTTAC AACAGTGGTA	550
	600
GAGAGCCGTC AACCGTGCAG ACACGGCCAA GGAGGTTTCC TTTGATGTGG	650
CTCTCGGCAG TTGGCACGTC TGTGCCGGTT CCTCCAAAGG AAACTACACC	700
	750
GCTGCCCAA GACGGCCTTC ATCACCAACT TCACCTTGAC CATCGACGGT	800
CGACGGGTT CTGCCGGAAG TAGIGGTTGA AGTGGAACTG GTAGCTGCCA	850
attearter ataatet reegenete ateataan antataan tateat	900 🗸
01180.180. 0100088101 08601000.8 80880.08018	050

SYBR Green I Primer for Quantification

Automatically Design

進入 Primer Express 3.0 軟體

 $File \rightarrow New \rightarrow$ 選擇"TaqMan MGB Quantification 或 TaqMan Quantification" $\rightarrow OK$



Tools→按"Add DNA File" 1, 尋找序列存取位置,按下"Add",將序列檔案加入空白文件。亦

可在"Sequence" Tab 中使用 Copy & Paste 轉貼或直接輸入序列。



* Primer Express Software 只能接受.dan, .txt, .ab1,或.abi 的檔案格式,請事先將欲分析的序列存成純文字檔即可 (若序列是從 database download 下來時,請刪除與序列無關之資料)



可勾選" Double Strand", 即會顯示 double stranded DNA sequence

III Primer Express 3.0		
<u>File Edit View T</u> ools <u>W</u> indow Help		
TaqMan® MGB Quantification # 1		"×
Sequence Parameters Primers / Probes Order		
File Name NM_002217.txt		
Length 2809 bp. Selection 1 to 1 🔽 Double Stranded		
<u>, , , , , , , , , , , , , , , , , , , </u>		
TCAGCGAIGG CATTIGCAIG GIGGCCCIGI CICAICIIGG CICIGCICIC	50	
AGTCGCTACC GTAAACGTAC CACCGGGACA GAGTAGAACC GAGACGAGAG	100	
	150	
CAGCTTGGCA GCCTCTGGCT ICCCGAGAAG CCCCTTICGG CIGCTIGGGA	200	
GTCGAACCGT CGGAGACCGA AGGGCTCTTC GGGGAAAGCC GACGAACCCT	250	
	300	
AACGGAGCCT CCCGGAAGGG GTGGCCAATG GCATCGAGGT CTACAGTACC	350	
TTGCCTCGGA GGGCCTTCCC CACCGGTTAC CGTAGCTCCA GATGTCATGG	400	
	450	
RARAICARCI CCRAGGIGAC CICCCGIIII GUICACARIG IIGICACCAI	500	
TITINGTON GOLICCACIO GAGGGCANAN CONGLETINC NACNOLOGIN	550	
CARAGECERTE ANECESTICES ACACESSICAN CRACETTEC TITENTERS	600	
CTCTCCCCCC TACCCCCCCC TACCCCCCCC CTCCCCCCCC	650	
	700	
AGTGCTCAA GACGGCTTC ATCACCAACT TCACCTTGAC CATCGACGGT	750	
TCGACGGGTT CTGCCGGAAG TAGTGGTTGA AGTGGAACTG GTAGCTGCCA	800	
	850	
GTTACCTACC CTGGGAATGT CAAGGAGAAG GAAGTTGCCA AGAAGCAGTA	900	~
To find Primers & Probes, click the "Find Primers/Probes" button	_	_

Tools 上的選項

1. Exclude:不必要的序列可利用"Exclude Selected Bases" 將序列刪掉

- 先選取不要的序列範圍,點選 "Exclude",即可看見此區域被劃掉

	🗋 😂 🖥 🖗 🕺 🛍 🗋 🔁 📕 🕨 🖪 🔄 🕂 🖬 🚽 🔶 💷		
۱	TaqMan® MGB Quantification # 1		J 🗙
ľ	Sequence Parameters Primers / Probes Order		
	File Name NM_002217.txt		
	Length 2809 bp. Selection 1 to 50 🔽 Double Stranded		
	YY	uulu	
	ICAGCGAIGG CATTIGCAIG GIGGCCCIGI CICATCIIGG CICIGCICIC	50	
	AGTEGETACE GTAAACGTAC CACCGGGACA GAGTAGAACE GAGACGAGAG	100	
		150	
	CAGCTIGGCA GCCTCIGGCI ICCCGAGAAG CCCCTIICGG CIGCIIGGGA	200	
	GTCGAACCGT CGGAGACCGA AGGGCTCTTC GGGGAAAGCC GACGAACCCT	250	
		300	
	AACGGAGCCI CCCGGAAGGG GIGGCCAAIG GCAICGAGGI CIACAGIACC	350	
	TIGCCICGGA GGGCCIICCC CACCGGIIAC CGIAGCICCA GAIGICAIGG	400	
	**************************************	450	
		500	
	IIIIAGIIGA GOIICCACIO GAGOGCAAMA COAGIGIIAC AACAGIGGIA	550	
	CARAGENETE AACENTERAA AEACENTERA GRAGETTEE TITRATENS	600	
		650	
		700	
	AGTECCEAL BACEGOCTTC ATCACCAACT TCACCTERSC CATCERCERT	750	
		800	
I	TOUCOOOTT CLOCCOOUND TUDIOOTTOU UNITOOUUCID ATUACIACCU	850	
I	ALLARCE CLARKER CONCERNENCE OF A C	900	~
1	MUTRALINA, AURIOPHILI AOPANOVOV VOCULINAA, AVOINAVITO	050	



2. Junction:如果已經知道序列上 junction 位置則可利用"Junction" J

- 框選 juction 的位置(必須含 2 bases), 再點選 "Junction", 即可看見此區域被紅色標

記起來。	
🗋 😂 🛃 😂 X 🗎 🗋 🗶 🔳 🕨 🛋 🔿 🗲 🗉 🛄 💩 🞯 🥥 🛒 🤒	
📓 TaqMan® MGB Quantification #1 Junction	_ 7 🛛
Sequence Parameters Primers / Probes Order	
File Name NM_002217.txt	
Length 2809 bp. Selection 289 to 290 🗹 Double Stranded	
L	
TCAGCGATGG CATTTGCATG 31GGCCCTGT CTCATCTTGG CTCTGCTCTC	50 🔼
AGTCGCTACC GTAAACGTAC <mark>CA</mark> CCGGGACA GAGTAGAACC GAGACGAGAG	100
	150 📃
CAGCTTGGCA GCCTCTGGCT TCCCGAGAAG CCCCTTTCGG CTGCTTGGGA	200
GTCGAACCGT CGGAGACCGA AGGGCTCTTC GGGGAAAGCC GACGAACCCT	250
	300
AACGGAGCCT CCCGGAAGGG GTGGCCCARUS GCATCGAGGT CTACAGTACC	350
IIGCCICEER EGECCIICCC CACCEGI <mark>TAC</mark> CEIRECICCA GAIGICAIGE	400
*****	450
MAMAILARUI UUMAUUUKU CIUUUUIIII UUUAUMAID IIGIUMUMI	500
	550
angharraty hereatacha hereascan asharttee titantatas	600
	650
	700
AGCTGCCCAA GACGGCCTTC ATCACCAACT TCACCTTGAC CATCGACGGT	750
TCGACGGGTT CTGCCGGAAG TAGTGGTTGA AGTGGAACTG GTAGCTGCCA	800
	820
GTTACCTACC_CTGGGAATGT_CAAGGAGAAG_GAAGTTGCCA_AGAAGCAGTA	900

3. 在"Parameters" Tab 中可看到 Primer Tm 值設定及其他 Primers 設計之規範

Sequence Parameters Primers / Probes Order		
Recenter	. Velue	
Parameter	Value	
		^
	80	
Max Primer Tm	60	
Max Difference in Tm of Two Primers	2	
Primer GC Content		
Min Primer %GC Content	30	
Max Primer %GC Content	80	3
Max Primer 3' GC's	2	
Primer 3' End Length	5	
Primer 3' GC Clamp Residues	0	
Primer Length		
Min Primer Length	9	
Max Primer Length	40	
Optimal Primer Length	20	
Primer Composition		
Max Primer G Repeats	3	
Max Num Ambig Residues in Primer	0	
Primer Secondary Structure		
Max Primer Consec Base Pair	4	
Max Primer Total Base Pair	8	
Primer Site Uniqueness		
Max % Match in Primer	75	
Max Consec Match in Primer	9	
Max 3' Consec Match in Primer	7	×



回到 "Sequence" Tab, Tools → Find Primers/Probes , 軟體即開始找尋適當的 Primers/Probe pairs

a,	Can	Adate Primers &	Pubei												
		Fred Start	FedLen	FedTm	Feed 1GC	Rev Stat	Revien	Rev Tm	Rev MC	Probe Stat	Probe La	Probe Tm	Probe 10C	I A	
	T	1343	15	59	60	1295	15	10	57	1,250	17	710	69	82	
	2	1040	19	58	58	1099	22	59	45	1060	17	68	41	79	
	3	1040	39	58	58	1100	22	59	45	1060	17	68	41	80	
	4	1043	19	58	58	1100	22	59	45	1060	18	69	29	80	
	5	1040	19	58	58	1100	22	59	45	1061	17	69	41	80	
	<u>8</u>	1822	21	58	17	1987	20	60	55	1953	14	68	71	84	
	2	1822	21	58	\$7	1987	19	58	58	1953	14	68	71	84	
	8	1822	21	58	82	1898	18	59	63	1853	14	68	21	85	
	9.	1822	21	58	52	1892	19	69	63	1853	14	68	21	84	
	10	2169	21	56	57	2245.	20	59	55	2204	15	69	67	84	
	11	2169	21	58	57	2245	20	59	55	2206	16	69	69	84	
	12	2169	21	58	57	2245	20	59	55	2209	15	69	73	- 84	
	13	2163	21	58	57	2245	20	59	55	2210	14	68	79	- 84	
	14	2163	21	58	57	2245	20	59	55	2218	15	70	73	84	
	€.									- A.M.	200	1942	1946		
C Location															
														_	
)	Seco	ondary Structure													
	1		Oligo			Length		Harpin Set Daves Cross Daves							
	0	Forward Prin	er :			15	15 Nott Stable Structure Found								
	0	Reverse Prim				19		2000ITT 5'							
	0	Probe				17		4 III							
	Fr	rward Presen						4000CX	III F						
	T	TECCCEPCE	ATTT												
	0.	evenue Preser													
Revenue Plater															
	GAAGCECTGCAACTGCAAA														
	Fobe														

Primer Express 軟體會找到 Candidate Primers & Probe pairs, 一次 search 最多能找到 50 種組合,這 些組合列於"Primers / Probes" Tab 中,請注意若是進行 SYBR Green primer 設計,不需看 probe 序列,只需参考 primer 序列組合。中間 "Location"說明 primers & probes 在序列中的相對位置,在 横線上方的數字代表起始位置,横線下方則代表終止位置。

"Sequence" Tab 中會顯示出與"Primers / Probes" Tab 相對應的一對 Primers/Probe: 粉紅色片段是 Probe 的位置,藍色代表 Forward Primer,黄色片段則為 Reverse Primer,如下圖所示。(*但並不表 示此為最佳的設計)

🧱 TaqMan® MGE	Quantification	≇1							
Sequence, Paramet	ers Primers / Prob	es Order							
File Name NM	I_002217.txt								
Length 28	09 bp. Selection	289	to	289	✓ Double Strar	nded			
<u>timuuluuu</u>	mhnunnin	muntu	սուրը		hummutur	uuuluu	աստեստ	minim	uhunununun
CATCOCCCC AACTT	COOT TOTATAACOT	aggettagg	AACAATCTC						
CATCOGGGGC AAGIIC	GCCI IGIAIAACCI	CCCCANACCC	TTOTTACACI	1 F					
BINGCCCCC0 IICAN	0000A ACAIAI100A	CCCOMMACCO	IIIIIIAGAC.						
ATTATAACTT CCTGG/	AGAAC ATGGCCCTGG	AGAACCATGG	GTTTGCCCGG	3					
TAATATTGAA GGACC?	CTTG TACCGGGACC	TCTTGGTACC	CAAACGGGCC						
CGCATTTATG AGGACT	ICTGA TGCCGA <mark>TTTG</mark>	CAGTTGCAGG	GCTTCTATG/	λ.					
GCGTAAATAC TCCTG/	AGACT ACGGCT <mark>AAAC</mark>	GTCAACGTCC	CGAAGATACI	C.					
GGAGGTGGCC AACCC/	ACTGC TGACGGGTGT	GGAGATGGAG	TACCCCGAG	۹.					
CCTCCACCGG TTGGGT	IGACG ACTGCCCACA	CCTCTACCTC	ATGGGGCTCI	C					
				8					
ACGCTATCCT GGACCI	CACC CAGAACACTT	ACCAGCACTT	CTACGATGG	3					
TGCGATAGGA CCTGG/	GTGG GTCTTGTGAA	TGGTCGTGAA	GATGCTACCO	3					
TOTGAGATOG TOOTO	CCGG GCGCCTCCTC	GACGAGGACA	TGAACAGCTI	r.					
AGACTETAGE ACCAC	adda adadaadaa	CTGCTCCTGT	ACTTGTCG4						
NUNCICINUC ACCAC		CIOCICCIOI	ACTIVICUM	2					~



在 "**Primers / Probes**" Tab 中會將每對 Primers/Probe 的組合列出來,請從中挑選出適當的組合,挑選方式可依據第 2 頁 primer/ probe design guideline。

* SYBR Green primer 設計必須要注意避免有 primer dimer 的產生

-在"**Primers / Probes**" Tab 中任選一組 primer set,從右下方來觀察 Primer Secondary Structure, 則可得之 Hairpin, Self Dimers 及 Cross Dimers 的情況,選擇鍵結數越少越好的組合,其中又以 GC 的鍵結比例越少越好。

Seq	ueno	e Parameters	Primers / Prob	He Order												
0	Carv	5date Primers 8	Probes													
	=	Feed Start	FwdLen	Fwd Tm	Fwd %GC	Rev Start	Rev Len	Rev Tm	Rev %GC	Probe Start	Probe Le	Probe Tm	Probe %GC	An		
	1	1343	15	59	60	1395	19	59	53	1359	17	70	59	82 ^		
	2	1040	19	58	58	1099	22	59	45	1060	17	68	41	79		
	3	1040	19	58	58	1100	22	59	45	1060	17	68	41	80		
	4	1040	19	58	58	1100	22	59	45	1060	18	69	39	80		
	5	1040	19	58	58	1100	22	59	45	1061	17	69	41	80		
	6	1822	21	58	57	1887	20	60	55	1853	14	68	71	84		
	7	1822	21	58	57	1997	19	58	58	1953	14	68	71	84		
	8	1822	21	58	57	1888	18	59	61	1853	14	68	71	85		
	9	1822	21	58	57	1892	19	59	63	1853	14	68	71	84		
	10	2169	21	58	57	2245	20	59	55	2204	15	69	67	84		
	11	2169	21	58	57	2245	20	59	55	2206	16	69	69	84		
	12	2169	21	58	57	2245	20	59	55	2209	15	69	73	84		
	13	2169	21	58	57	2245	20	59	55	2210	14	68	79	84		
	14	2169	21	58	57	2245	20	59	55	2210	15	70	73	84 🚩		
	<													>		
0	Loca	ilion														
	_					10-10-00										
_																
9	240	inday studu	e						-	_						
			Oligo			Length		Haipin Sell Dimers Cross Dimers								
	۲	Forward Pri	ner			19		Most Stable Structure Found								
	0	Reverse Pris	ner			22		1. Forward and Reverse Cross Dimer								
								5' ADDADDCCADDACUTTTUT 3'								
	0	Probe														
	Forward Primer					3. CORTCATEGAEGITOGECATEC 2.										
AGGAGGCCAGGACGTTTGT																
	B	everse Primer						2. F	orward and Pr	obel Cross Bi	1001					
	0	CGTCATTGAT	GTTGGTCATT	Ċ				5' AO	AGCATOGAGGATA	AA 3'						
	P	obe														
	A	GAGCATGGA	IGATAAA						3" TOT	TTOCADGACCOGA	05A 51					



決定 primers set 之後則可進行存檔,存檔的方式從 File → Save As 存檔。

如果想將所選擇的 primers set 單獨儲存,可利用 Export → Order Info...的方式,或者要儲存 50 個 Primers/Probes 清單,可點選 Export → Primers/Probes List...,以上兩種方式都可存成.txt 檔案,在 Excel 中開啟。



Manually Design

當軟體無法自動尋找到 Primer 組合時,先確定 Primer 想要置放的位置

開啟一個新的 Quantification Document, 並把欲設計的序列檔案加入 (參照第 13 頁)。

1. Forward Primer 設計:

在"Sequence" Tab 中將預定 Primer 序列 highlight 起來(至少 25 bases 之長度,長度依序列結構而定) 後,先利用 Edit 中 Copy (Ctrl+C)之功能複製序列,再依下圖至 Tools → Primer Probe Test Tool 中, 選擇欲設計的 document type (即"TaqMan MGB Quantification 或 TaqMan Quantification"),並確認 Parameter 設定為"Default",利用 Paste (Ctrl+V) 將序列貼在 Fwd Primer 欄位,從右邊即可觀察測 試序列之 Tm,%GC 和長度是否合適。

Sequence F	Parameters P	himens / Phobe	ss Order									
🛐 File Nan	ne NM_0022	17.od			2 Primer Probe Test	Tool						X
Length	2809 bp.	Selection	175	to 15	Parameters							_
X					Document Type: Tag	fan® MGB Quantification	Pari	ameter: Default		<u> </u>	Вюние	
CARCITERICA	OCCTCTOSCT	TCCCGAGAAS	COCCITIONS	CTOCTTOOGA	Primers and Probes							
GTOGAACOST	CIGAGACCIGA	ASSOCICTIC	0003AAA0000	GACGAACOCT								
					Fwd Primer	CGTTTTGCTCACAATGTT	IGTCA		Tm	%GC	Length	
AACGGAGCCT	CC03644693	GEGGCCAAEG	GCATCGAGGT	CTACASTACC			N		58.3	41	22	
TISCCICOSA	0800CTTCCC	CACCOUTTAC	CSTASCTCCA	GATETCATES	Rev Primer		18		Im	AGC 0	Length	
*******	antenana	OT COMPTEN	COTOLOGIATO.	TRATALOUT					100	0	Locath	
TTTTATTO	OCTABOLISMC OCTABOLISMC	Choodenana	OCICACAA10	ANDAGENCIAL	Probe 1				70.0	703L	Length	
TTTWOTION	dell'ecorcio		CONSTOLING	MUNIEUULA					Te	700	Landh	
SIGNOCOTTC.	ANOTSTOCKS	ACACORCEAN	OBIOSTITIC:	TTEMPETOR	Probe 2				0.0	0	Longen 0	
CTCTOSGCAG	TTOCACSTC	TOTOCCOUTT	CCTCCAAAGG	AAACTACACC	Tim						<u> </u>	
				THE REAL PROPERTY AND A DECIMAL OF A DECIMALO OF A DECIMALO OF A DECIMALO OF A DECIMAL OF A DECIMAL OF A DECI								
AGCTOCCCAR	GAODSOCTTC	ATCACCAACT	TOACCTEGAC	CATOGACOUT	-Secondary Structure							
TOSACSBOTT	стоссозька	TASTOSTICA	ASTOSAACTS	STARCERCCA	Secondary structure	1		TRANSMERSON DRAWN	Correction of the			
					Oligo	Length		Harph Der Dinier	Cross Dimers			
OTTACCTACC.	CT008AAT0T	CANOGNOANS	GAAGTTOCCA	AGAAGCAGEA	Forward Primer	22						
CARTOGATOG	GACCETTACA	OLICICICIC	CITCAACOOT	TCITOSTCAT	Reverse Primer	0						
					OProbe 1	0						
TGAAAAGOCT	GIGLCCCW03	OCANGACOGC	COCTIONIC	AAGOCCTCTG	O Probe 2	0						
ACTITICOBA	CACABBITICC	ONITCIBCCE	GCCGAACCAG	TECCOGRAGAC	(
					Show	secondary Structure						
GGAGGAAGTT	GENGARGITC	ACAGICIO36	TCAACOTOOC	10CA09CA9C			_					
CITCHICAN	cerencows	TOTOWSNOCC	Not Facación	wostocates								1300
AAASTCACCT	TOBASCTARC	CTACOMONS	стостакках	0004044008								1350
TTECACTOGA	AGCTOGATTO	GATOCTOCTC	GAOGACTECT	CONTRITICCC								1400
		**********	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~									1450

確認 Forward Primer 的 Tm 值在 58-60℃,如果 Tm 值不符合,可直接在 Fwd Primer 中框選不同的 序列片段,並觀察右邊對應的 Tm,%GC 和長度,以找出最適當的 Primer (請參照 primer/ probe design guideline),若框選到適合的片段,點一下"Trim",就可以將未框選的序列直接刪除,只留下需要 的序列。

Primers and Probes —				
Fwd Primer	CTCCCGTTTTGCTCACAATGTTGTCA	Tm 58.3	%GC 41	Length 22
Rev Primer		Tm 0.0	%GC 0	Length 0
Probe 1		Tm 70.0	%GC 0	Length 0
Probe 2		Tm 0.0	%GC 0	Length 0
Trim				



2. Reverse Primer 設計:

在"Sequence" Tab 中將預定的 Reverse 序列 highlight 起來(至少 25 bases 之長度,長度依序列結構而 定)後,然後利用 Edit →" Copy Complement "將序列貼到 Tools → Primer Probe Test Tool 中的 Rev Primer 欄位。

Sequence Parameters Primers / Probes Order		
🔞 File Name NM_002217.txt	Rimer Probe Test Test	N N
Length 2809 bp. Selection 316 to	Parametera	
	Document Type: TagMan® MGB Quantification V Parameter: Default	Urowse
CASCITIGGCA GCCICIGGCI ICCCGAGAAG CCCCITICOS CIGCIIGOS	Polyana and Darker	
STCGAACOST OSBAGACOSA ASSOCTOTTO GOSGAAASOO GACGAACOO	Primers and Prodes	
	European COLLECTION ALGUERA	Tm %6C Length
ARCHARDET CECCURAGES STORESALS CELEBRATIC		58.3 41 22
HIGCHCOM GEOCHTCCC CROSELING COLMECTICA GALORCARE	Rev Primer TTCTTGGCAACTTCCTTCTCCTTGACATT	Tm %6C Length
AAAATCAACT COAASSTORC CTCC COLLEGE SHOWAATS INSURACCA		56.6 41 23
TTITAGITGA OSTICCACIG GAOL <mark>ICAAAA</mark> COASTOTTAC AACAGI <mark>D</mark> IJI	Probe 1	70.0 0 0
		Tn %5C Length
GAGAGCOSTC AACOSTOCAG ACACOGOCCAA GGAGSTITICC TITGATGIG	Probe 2	0.0 0 0
CTCTOSSCAS TISSCAOSIC ISTOCOSSTI CCICCAAASS AAACIACAC	Trim	
ANTIGODAL GROSSOTTIC ATCHORAGT TOMOTTORC CATORAGE		
TOSACOBSTT CTOCCESAAS TASTOSTTGA ASTOSAACTS STACTOC	Secondary Structure	
	Oligo Length Halpin Set Dimers Dx	iss Dimers
OTTACCTACC CTODGAATOT CAAGGAGAAG GAAGTTGCCA AGAAOCAGE	Forward Primer 22	
CAADOSATOS GACOCITACA GITCCTCITC CITCAACOOI TCITCSTCA	O Reverse Primer 29	
	O Probe 1 0	
IGAMMAGET GIGICCCAGE GCAGACGOC COGCITEGIC AMBRICIC	OProbe 2 0	
WITTECOM CACADOTEC COLICIDECO OCCAMACIÓN IECOGRAM	Charles Concerden Churchen	
GUAGUAAUTT GUAGUAUTTC ACAUTCICOU TCAACUTODC TGCAGUCAU	Show secondary secondary secondary	
OCTOCITICAA OCTOTICAAS ISTCASASCO ASTISCACOS ACSICOSTO		1300
		1100

確認 Reverse Primer 的 Tm 值也能符合在 58-60℃,如果 Tm 值不符合,可直接在 Rev Primer 中框 選不同的序列片段,並觀察右邊對應的 Tm,%GC 和長度,以找出最適當的 Primer (請參照 primer/ probe design guideline),若框選到適合的片段,點一下"Trim",就可以將未框選的序列直接刪除, 只留下需要的序列。

-Primers and Probes—				
Fwd Primer	CGTTTTGCTCACAATGTTGTCA	Tm 58.3	%GC	Length
Rev Primer	TTCTTGGCAACTTCCTTCTCCTTGACATT	Tm 57 G	%GC	Length
Probe 1		Tm	43 %GC	Length
Durba 2		70.0 Tm	0 %GC	0 Length
Probe 2		0.0	0	0



3. 在 SYBR Green primer 設計中必須要注意避免有 primer dimer 的產生

-在 Primer Probe Test Tool 中選定 primer set 後,點選 "Show Secondary Stucture",即可從右下方來 觀察此組 Primers 的二級結構,如 Hairpin, Self Dimers 及 Cross Dimers 的情況,避免鍵結數太多, 其中又以 GC 的鍵結比例越少越好,如果此組 primer 二級結構嚴重,請重新尋找適當位點。

🌆 Primer Probe Test	Tool								
Parameters									
Document Type: Tag	Man® MGB Quantification 🛛 💽 F	^D arameter:	Default		~ [Browse			
Primers and Probes-									
Ewd Primer				Tm	%GC	Length			
rwurnnei	carrinacicadarariaria			58.3	41	22			
Dev Direct	COMPTONIC			Tm	%GC	Length			
Hev Primer	GCAACTTCCTTCTTCCTTGACATT			57.6	43	23			
				Tm	%GC	Length			
Probe 1				70.0	0	0			
				Tm	%GC	Length			
Probe 2				0.0	0	0			
Trim					·				
Secondary Structure	•								
Oligo	Length	Hairpin	Self Dimers Cr	ross Dimers					
• Forward Primer 22			Most Stable Structure Found						
O Reverse Primer 23			1. Forward and Reverse Cross Dimer						
O Probe 1 0			CGTTTTGCTCAC	AATGTTGTCA	3'				
O Probe 2 0									
Show	Secondary Structure	3'	GCAACTTCCTTC	TCCTTGACATI	5'				

當 Fwd Primer 序列確定之後,回到"Sequence" Tab 中將確定序列 highlight 起來,再利用 "Forward

Primer" → 固定起來,此時的 Fwd Primer 位置會被標定成藍色。

😹 TagMan 🤁 MGB Quantification # 1		
Segurate Parameters Primers / Probes Order		
Re Name NM, 002217.bt		
Length 2803 bp. Selection 1/5 to 136 Update Standed		
1		****
CARCTIRICA RECTIRICT ICCOMMAND COCCITIONS CIRCINGA	200	^
STOSACOT OSMARCSA ASSOCICTE GSSAAASCC SACSAACCT	250	
	300	
ACOUNTECT COORDANIES CONCOMPT CONNECTOR CONCERNCE	350	
	400	
AMATCANCE COMPUTING CECCONTENT DETENDANTS EDUCATE	450	
TITIAJITGA GUTCCACTO GAUGUCARAR CONTIGENC ARCHITOTA	500	
	600	
GABAGCOTC AACCITECAS ACACEDECAS GEAGUTITEC TITEATUTES	650	
CECTOBECKS ITERECACITE INTERCONTE OCTOCAMARS AMACTACACE	700	
	750	
ANCTROCOMA SACRECTIC ATCACCAMET TEACETISAC EXTERNOLT	800	
TCGACGBETT CTGCCBGAAG TAGTGGTTGA AGTGGAACTG GTAGCTGCCA	850	
	900	
DESINCENCE CLOBERTER CONSIGNED SINCE CONSIGNED A	950	
CARDARIO BACCITACA DI ECICITE CITEMANDI FETEDICAT	1000	
TRAAMORT STUTCCARE COMPACING CONSTITUTE AND CONTENTS	1050	
ACTITICODA CACABRITO: CUTECTROOD GOODAACCAU TECOBRANC	1100	
	1200	
DIAGDAASTT GOMDAASTTC ACMUTCTODE TCAACUTOOC TOCMBEAGC	1250	
Conservation and several law a several law a several law and the s	1000	



當 Rev Primer 序列確定之後,回到"Sequence" Tab 中將確定序列 highlight 起來,再利用 "Reverse

Primer" ← 固定起來,此時的 Rev Primer 位置會被標定成粉色。

TaqMan® MGB Quantification # 1	- 6 🗙										
Sequence Parameters Privers / Probes Order											
Rie Name NM_002217.txt											
Length 2909 bp. Selection 316 to 339 🔽 Double Stranded											
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accances connects vances and accances	250										
	300										
	350										
Therefore operation construction and then	400										
AMANDARY COMPUTER COOPERATE DEPENDENT	450										
TITLAUTINA OUTICASTA GAODAAAA DAUTUTAS ASSAULTA	500										
	550										
BAGAGEOUTIC AACOUTICAA GAAGGECAA GAAGUTTICC TITGATUT00	600										
CTCTOBERS TEBERAGIC DEDECESTI CCTCHARGE AARCTACKC	200										
	750										
AGCTROCCAR GAORGOTTIC ATCACCATOL CATCOLOGIEST	800										
TCORCEGENTE CTOCCEGARG TRETERITOR ASTOGRACTE GTADCTOCCA	850										
	900										
STIACCIAC CIGONAISI CAASSASAS GAASIGOCA ASAACASIA	950										
CAADSADSS SACCETACA SITCEPETE CITCAAOSET ICTICSTCAT	1000										
	1050										
TRAAAA00CT GTUTCCCA00 GCAA0AC00C C00CTT0JTC AA00CCTCT0	1100										
ACTITICOSA CACAGOSTICE OSTICEDECES OCCOARCEAS TECEDARIAE	1150										
	1200										
BOADDAAGTT GARGAAGTTC ACAGTCTCOG TCAACGTODC TOCADDCADC	1250										
CEDETECAA CEDETECAAS DETECASASCE ASTESCACOS AGRECUTOS	1300										
	1 100										

決定 primer/ probe set 後可利用 Copy & Paste 功能轉貼到一個新的 text 檔,並 save 起來做為未來參考資料。另外,也可以把此次設計 document 進行存檔,存檔的方式從 File → Save As 存檔。

Elo Edit	View <u>T</u> ools	Window Help		
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Savg As.	CH+E	1756 🛃		
Export	·)	Selection 351 to 351 C Double Stranded		
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Pwt	CM+P		lu	
Ext		Nonciette annowate accordent	250	2
AAC035A000	T 000354403	GEORGEARD GENERALD CANDIDATE CRACHERCE	300	
TTOCCTCOS	A GOOCCTTCC	CACCOULTAC COLACIDOA GALUTCATO	350	
			450	
AMATCANC	T CCAAGOTGA	COCONTRA STOCAST TOTACON	500	
TITLAGIDS	A OUTTOCACT	GAGGOCAAAA COACTUTEAC AACAUTOTA	550	
			600	
GAGAGCOT	C AACCOTOCH	ACACONCA OBADITICC ITIADITIO	650	
CICICIOUCA	6 1100CAC65	ISBOORT CECONOS ANCINOC	700	
AGCTRODOCA	A GAOSSOCTE	ATCACCARCT TCACCTERAC CATCHARDET	750	
TCOACOOUT	T CTOCCOOAN	TANTOTTON ANTONAACTO UTACCOCCA	850	
			900	
OTTACCTAC	C CTOOS ATC	CA AGAAOCASTA	950	
CAADGGADG	IS GACCOTAC	STRUCTURE STRUCTURE OF TETTOSTOAT	1000	
			1050	
raunaac	T OTOTOCCAS	OCAMACODE CONCILIONE AMORETETI	1100	
ACTITICOS	N CACABOUTC	CHICTROCE BOODWACKS ITCOSEARC	1150	
034054407	T OLASAOTT	ACMITETORI TEAADITRIC TREADEADE	1200	
OCTOCTICA	A OCTOTICAN	TREAMAGE ANTICACCO ADDRESS	1250	
			1350	
AAAGTCACC	T TOSAGCTAN	CTACGAGGAG CTOCTGAAGA GBCACAAGGG	1400	
TTTCA0T03	A ACCOUNTS	GAEGCTOCTC GAOGACETICT CONTUETCCC	1450	
			1500	¥
Sector Sector Sector				-

Primer Express Operation Guide

Primers & Probes for Allelic Discrimination

Automatically Design

進入 Primer Express 3.0 軟體

File→ New→選擇"TaqMan MGB Allelic Discrimination 或 TaqMan Allelic Discrimination"→ OK。 建議使用 TaqMan MGB probe 設計,以達到更佳效果。

I ype: TagMan® MGB Guantification	~
TagMan® MGB Quantification	
TaqMan® Quantification	
TagMan® MGB Allelic Discrimination	
TaqMan® Allelic Discrimination り	
OK Canc	el

Tools→按"Add DNA File" 🚺,尋找序列存取位置,按下"Add",將序列檔案加入空白文件。亦

可在"Sequence" Tab 中使用 Copy & Paste 轉貼或直接輸入序列。



^{*} Primer Express Software 只能接受.dan, .txt, .ab1,或.abi 的檔案格式, 請事先將欲分析的序列存成純文字檔即可

⁽若序列是從 database download 下來時,請刪除與序列無關之資料)



標定 SNP 位點

將 SNP 位點 highlight, Edit → Annotate → "SNP Target" ,選擇此 SNP 位點的變異型(variant)。

File Edit View Iools Window Help Image: Second Sec	
Image: Sequence Parameters Primers / Probes Order	
TaqMan® MGB Allelic Discrimination ≠ 2 V Sequence Parameters Primers / Probes Order	
Sequence Parameters Primers / Probes Order	
🔐 File Name 🛛 AY228765.txt	
Length 1548 bp. Selection 528 to 528 🗌 Double Stranded	
∇	
toona a baaraa daaraa daara c	adama
ATGGGAAATC CCCTCCAAAT CTCCATTTTC CTGGTGTTCT GCATCTITAT CCAATCAAGT GCTTGTGGAC AAGGCGTGGG AACAGAGCCC TTTGGGAGAA	100
GCCTTGGAGC TACTGAAGCT AGCAAGCCAT TAAAGAAGCC AGAGACCAGA TTCTACTTCT TCCAAGATGA AAACGATCGC CTGGGCTGTC GTCTCAGACC	200
TCAGCACCCG GAAACACTGC AGGAGTGTGG CTTCAACAGC TCTCAG M SNP/Target TGGTCGGTGG ATGGCTTGCT AGAAAACTGG	300
ATCTGGAAGA TAGTGAGTGC GCTGAAGTCC CGACAGTCCC AACCTG	400
CTGTTCAAAA CACCCGTATI GTGGGCCAGG ACGTGGCTGC TCTTCT	500
STACAGECTE GGAGEGEAGE TETCAGGETT CGCAGGEAGE TECATGE	600
TITGAGGGAA CGTCCCCCAA CGAGCGCCTT TCTCCTGATG ATGCCAA	700
GCATCAAGCA GCCCATTGCC CACTATGACT TCTACCCCAA CGGGGGG	800
тересталас вселтлаесс абассатела атегессат бабебе (С) СССССССССССССССССССССССССССССССССС	900
TTCCAGTGCA GCGACATGGG CAGCTTCAGC CAAGGTCTAT GCCTGA	1000
CAGGCAAGAG CAAGAGGCTC TTCCTCATCA CGCGAGCCCA GTCTCC	1100
GCCGGTAGAG CCTACTTTA CCATGTCGCT GCTGGGAACA AAAGAAG	1200
TATTCCTTCC TTATCACACT GGACAAAGAC ATCGGCGAGT TGATCC	1300
TGCAGACCAT CATGCTATGG GGCATAGAAC CTCACCACTC TGGCCTCATT CTGAAGACCA TCTGGGTCAA AGCTGGAGAG ACGCAGCAAA GAATGACATT	1400
TTGCCCCGAA AATCT6GATG ACCTCCAGCT TCACCCGAGC CAGGAGAAAG TCTTTGT6AA CTGTGAAGTG AAGTCAAAAA GACTGACTGA ATCGAAAGAG	1500
CAGATGAGTC AAGAGACCCA TGCAAAAAAA TAAAGAAGTC TATTCTTT	1548
	1040

例如:在 528 位置是 G/A 的 SNP,點選 G 和 A 之間的" \mathbf{R} ",然後按下 OK, SNP 位點即被標定成 紅色小寫的 r。

	Sequence	Parameters F	Primers / Probe	es Order							
	🔐 File Nan	ne AY22876	5.txt								
	Length	1548 bp.	Selection	529	to !	529 📃 Do	ouble Strander	1			
	L										7
	ATGGGAAATC	CCCTCCAAAT	CTCCATTTTC	CTGGTGTTCT	GCATCTTTAT	CCAATCAAGT	GCTTGTGGAC	AAGGCGTGGG	AACAGAGCCC	TTTGGGAGAA	100
	GCCTTGGAGC	TACTGAAGCT	AGCAAGCCAT	TAAAGAAGCC	AGAGACCAGA	TTCCTGCTCT	TCCAAGATGA	AAACGATCGC	CTGGGCTGTC	GTCTCAGACC	100
	TCAGCACCCG	GAAACACTGC	AGGAGTGTGG	CTTCAACAGC	TCTCAGCCGC	TTATCATGAT	CATCCACGGG	TGGTCGGTGG	ATGGCTTGCT	AGAAAACTGG	200
	ATCTGGAAGA	TAGTGAGTGC	GCTGAAGTCC	CGACAGTCCC	AACCTGTGAA	TGTGGGGTTA	GTGGACTGGA	TCTCCCTGGC	ATACCAGCAC	TACACCATTG	400
	CTGTTCAAAA	CACCCGTATT	GTGGGCCAGG	ACGTGGCTGC	TCTTCTCCTA	TGGCTGGAGG	AATCTGCGAA	GTTTTCTCGG	AGCAAAGTTC	ACCTAATTGG	500
	GTACAGCCTG	GGAGCGCACG	TCTCAGG <mark>T</mark> TT	CGCAGGCAGC	TCCATGGACG	GGAAGAACAA	GATTGGAAGA	ATCACAGGGC	TGGACCCTGC	GGGCCCTATG	600
	TTTGAGGGAA	CGTCCCCCAA	CGAGCGCCTT	TCTCCTGATG	ATGCCAATTT	TGTGGACGCC	ATTCATACCT	TTACCAGGGA	GCACATGGGC	TTGAGTGTGG	700
	GCATCAAGCA	GCCCATTGCC	CACTATGACT	TCTACCCCAA	CGGGGGGCTCC	TTCCAGCCTG	GCTGCCACTT	CCTGGAACTC	TACAAACACA	TTGCAGAGCA	800
	TGGCCTAAAC	GCCATAACCC	AGACCATCAA	ATGTGCCCAT	GAGCGCTCCG	TGCACCTCTT	CATTGACTCC	TTGCAACACA	GTGACCTGCA	GAGCATCGGC	900
l	TTCCAGTGCA	GCGACATGGG	CAGCTTCAGC	CAAGGTCTAT	GCCTGAGCTG	CAAGAAGGGC	CGTTGCAACA	CTCTGGGTTA	TGACATCCGC	AAGGACCGGT	1000
	CAGGCAAGAG	CAAGAGGCTC	TTCCTCATCA	CGCGAGCCCA	GTCTCCCTTC	AAAGTTTATC	ATTACCAGTT	CAAGATCCAG	TTCATCAATC	AAATTGAGAA	1100
	GCCGGTAGAG	CCTACTTTTA	CCATGTCGCT	GCTGGGAACA	AAAGAAGAAA	TAAAGAGAAT	TCCCATCACC	CTGGGCGAAG	GAATTACCAG	CAATAAAACC	1200
	TATTCCTTCC	TTATCACACT	GGACAAAGAC	ATCGGCGAGT	TGATCCTGCT	CAAGTTCAAG	TGGGAAAACA	GTGCAGTGTG	GGCCAATGTG	TGGAACACAG	1300
	TGCAGACCAT	CATGCTATGG	GGCATAGAAC	CTCACCACTC	TGGCCTCATT	CTGAAGACCA	TCTGGGTCAA	AGCTGGAGAG	ACGCAGCAAA	GAATGACATT	1400
	TTGCCCCGAA	AATCTGGATG	ACCTCCAGCT	TCACCCGAGC	CAGGAGAAAG	TCTTTGTGAA	CTGTGAAGTG	AAGTCAAAAA	GACTGACTGA	ATCGAAAGAG	1500
	CAGATGAGTC	AAGAGACCCA	TGCAAAAAAA	TAAAGAAGTC	TATTCTTT						1548
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a lenc	a Parameters	Priners / Prot	et Dider										
Can	didate Primers I	Puber R											
=	Fwid Start	Fed Lon.	FedTm	Fwd %GC	Rev Stat	Rev Len	BerTm	Rev 46C	Probe1 5	Probe1 L	Probel Tm	Probel S.	1P
1	077	25	10	44	560	20	60	50	521	15	15	60	51
2	477	23	59	43	(560	20	60	50	521	16	66	63	5
3.	477	23	59	43	560	20	60	50	522	15.	65	67	5
4	475	23	58	43	560	20	50	50	521	15	65	60	5
5	475	23	58	43	560	20	60	50	521	16	66	63	5
6	475	29	58	43	560	20	00	50	522	15	65	07	5
7	475	24	59	42	560	20	60	50	521	15	65	60	5
8.	475	24	59	42	560	20	60	50	521	16	66	63	5
3	4/5	24	59	42	560	20	60	50	522	15	65	67	5
10	474	23	59	43	560	20	60	50	521	15	65	60	5
11	474	23	59	43	560	20	60	50	521	16	66	63	5
32	474	23	59	43	560	20	60	50	522	15	65	67	5
88	474	134	164.	147	3540	196	160	150	1658	114	「病	ish.	10
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Sec	ondary Structur												
		Oligo			Length		Haipin Se	Diment Cross	Dimena				
0	Forward Pri	ner			23		Host 2	table Structu	re Found				
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6	Probe 1				15		L.	111					
Ó	Probe 2				14		loctas	111					
THE PROPERTY	orward Prever CGGAGOWAL evente Prever TGTTCTTCCC tobe 1 CTCANDATTC	STCCATGGA	TQ.										

Tools → Find Primers/Probes ▶, 軟體即開始找尋適當的 Primers/Probes pairs

Primer Express 軟體會找到 Candidate Primers & Probes pairs, 一次 search 最多能找到 50 種組合,這些組合列於"Primers / Probes" Tab 中。中間"Location"說明 primers & probes 在序列中的相對位置, 在橫線上方的數字代表起始位置,橫線下方則代表終止位置。

"Sequence" Tab 中會顯示出與"Primers / Probes" Tab 相對應的一對 Primers/Probes: 粉紅色片段是 Probe 1 的位置,綠色片段則代表 Probe 2 的位置(2 個 Probe 重疊的序列會呈現綠色),藍色代表 Forward Primer,黃色片段則為 Reverse Primer,如下圖所示。(*但並不表示此為最佳的設計)

🗱 TaqMan® MGB Allelic Discrimination # 2	- 7 🛛
Sequence Parameters Primers / Probes Order	
🔞 File Name AY228765.txt	
Length 1548 pp. Selection 528 to 528 Double Stranded	
ATGGGAAATC CCCTCCAAAT CTCCATTITC CTGGTGTTCT GCATCTITAT CCAATCAAGT GCTTGTGGAC AAGGCGTGGG AACAGAGCCC TTTGGGAG/	VA 100
GCCTTGGAGC TACTGAAGCT AGCAAGCCAT TAAAGAAGCC AGAGACCAGA TTCCTGCTCT TCCAAGATGA AAACGATCGC CTGGGCTGTC GTCTCAGAG	3C 200
TCAGCACCCG GAAACACTGC AGGAGTGTGG CTTCAACAGC TCTCAGCCGC TTATCATGAT CATCCACGGG TGGTCGGTGG ATGGCTTGCT AGAAAACTG	3G 300
ATCTGGAAGA TAGTGAGTGC GCTGAAGTCC CGACAGTCCC AACCTGTGAA TGTGGGGTTA GTGGACTGGA TCTCCCTGGC ATACCAGCAC TACACCAT	(G 400
CIGTICAAAA CACCCGIAII GIGGGCCAGG ACGIGGCIGC ICIICICCIA IGGCIGGAGG AAICIGCGAA GIIIICICGG AGCAAAGIIC ACCIAAIIG	3G 500
GTACAGCCTG GGAGCGC <mark>ACG</mark> TCTCAGG <mark>TTT CGCAGGCAGC TCCATGGACG</mark> GGAAGAACAA GATTGGAAGA ATCACAGGGC TGGACCCTGC GGGCCCTAT	(G 600
TTTGAGGGAA CGTCCCCCAA CGAGCGCCC TCCCTGATG ATGCCAATTT IGIGGACGCC ATICAIACCT TTACCAGGGA GCACAIGGGC TTGAGIGIC	^{3G} 700
GCATCAAGCA GCCCATTGCC CACTATGACT ICTACCCCCAA CGGGGGGCTCC ITCCAGCCTG GCTGCCACTT CCTGGAACTC TACAAACACA ITGCAGAGG	X 800
TGGCCTAAAC GCCATAACCC AGACCATCAA ATGTGCCCAT GAGCGCTCCG TGCACCTCTT CATTGACTCC TTGCAACACA GTGACCTGCA GAGCATCGC	3C 900
TTCCAGTGCA GCGACATGGG CAGCTTCAGC CAAGGTCTAT GCCTGAGCTG CAAGAAGGGC CGTTGCAACA CTCTGGGTTA TGACATCCGC AAGGACCGG	3T 1000
CAGGCAAGAG CAAGAGGCTC TTCCTCATCA CGCGAGCCCA GTCTCCCTTC AAAGTTTATC ATTACCAGTT CAAGATCCAG TTCATCAATC AAATTGAG/	^{4A} 1100
GCCGGTAGAG CCTACTTTTA CCATGTCGCT GCTGGGAACA AAAGAAGAAA TAAAGAGAAAT TCCCATCACC CTGGGCGAAG GAATTACCAG CAATAAAAG	C 1200
TATTCCTTCC TTATCACACT GGACAAAGAC ATCGGCGAGT TGATCCTGCT CAAGTTCAAG TGGGAAAACA GTGCAGTGTG GGCCAATGTG TGGAACAC/	1G 1300
TGCAGACCAT CATGCTATGG GGCATAGAAC CTCACCACTC TGGCCTCATT CTGAAGACCA TCTGGGTCAA AGCTGGAGAG ACGCAGCAAA GAATGACA	(T 1400
TTGCCCCCGAA AATCTGGATG ACCTCCAGCT TCACCCGAGC CAGGAGAAAG TCTTTGTGAA CTGTGAAGTG AAGTCAAAAA GACTGACTGA ATCGAAAGA	NG 1500
CAGATGAGTC AAGAGACCCA TGCAAAAAAA TAAAGAAGTC TATTCTTT	1548

Primer Express Operation Guide

在 "**Primers / Probes**" Tab中會將每對Primers/Probe的組合列出來,請從中挑選出適當的組合,挑選 方式可依據第 2 頁primer/ probe design guideline。在MGB Probe的篩選中,亦可在"**Parameters**" Tab 中加選<u>"C"比"G"多的序列</u>作為進一步篩選的參數。

Sequence Parameters Primers / Probes Order	
Parameter	Value
Max Primer G Repeats	3
Max Num Ambig Residues in Primer	0
Primer Secondary Structure	
Max Primer Consec Base Pair	4
Max Primer Total Base Pair	8
Primer Site Uniqueness	
Max % Match in Primer	75
Max Consec Match in Primer	9
Max 3' Consec Match in Primer	7
Probe Tm	
Min Probe Tm	68
Max Probe Tm	70
Probe GC Content	
Min Probe %GC Content	30
Max Probe %GC Content	80
Probe Length	
Min Probe Length	13
Max Probe Length	25
Probe Composition	
Max Probe G Repeats	3
Max Num Ambig Residues in Probe	0
No G at 5' End in Probe	✓
Select Probe with more C's than G's	
Probe Secondary Structure	43
Max Probe Consec Base Pair	4
Max Probe Total Base Pair	8
Min Amplified Region Tm	0
Max Amplified Region Tm	85
Min Amplified Region Length	50
Max Amplified Region Length	150
🗖 General	
Max Primers / Probes	50

決定 primer/ probe set 之後則可進行存檔,存檔的方式從 File → Save As 存檔。

如果想將所選擇的 primer/ probe set 單獨儲存,可利用 Export → Order Info...的方式,或者要儲存 50 個 Primers/Probes 清單,可點選 Export →Primers/Probes List...,以上兩種方式都可存成.txt 檔案, 在 Excel 中開啟。

Elle	Edt	Vev	Tools	Window Help											
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	Doen.		CM+0							-					
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	lave		CM+S	nimers / Probes 0:	der										
	Lave A		CM+E	obes											
	larger 1			Semence	-	Fwd %GC	Rev Stat	Rev Len	Rev Tm	Rev 3GC	Probe1 S	Probe1 L.	Probe1 Tm	Probel X.	Pot
			C244+11	Paramatara	1	43	560	20	60	150	521	15	65	60	518
	rage :	eup	CH+0	Piner/Doha Lin	. ľ	43	560	20	60	50	521	16	66	63	518
	71N		C21+P	Printer/Price Lis		43	560	20	60	50	522	15	65	67	518
_	[xR			Urser Into	<u></u>	43	560	20	60	50	521	15	65	60	518
	5	4/5		23 58	-¢	43	560	20	60	50	527	16	66	63	518
	7	475		24 59		42	560	20	60	50	521	15	65	60	518
	8	475		24 59		42	560	20	60	50	521	16	66	63	519
	ž	475		24 59		42	560	20	60	50	522	15	65	67	518
	10	474		23 59		43	560	20	60	50	521	15	65	60	518
	11	474		23 59		40	560	20	60	50	521	16	66	ស	518
	12	474		23 59		43	560	20	60	50	522	15	65	67	518
	12	474		124 149		42	1460	120	IRN	190	1624	115	65	160	512
0	Loc	ation													
F	Sec	ondary	Shuchaw												
		,		Olice	_		Length		Haipin Se	# Dimers Cross	Dimers				
	0	Forw	ord Prim	er			23		Most S	table Structs	re Found				
	lõ	Reve	ree Prime	er .			20								
	lo	Probe	1				16		Heirpin	s not found					
	0	Probe	2				14								
	E DE	orward TCTCC leverse TGTTC hobe 1	Primer IGAGCAA Primer LTTCCCG	AGTTCACCTAA TCCATGGA											
	P	cobe 2	CAGGGT	TC	_										

Manually Design

當軟體無法自動尋找到 Primer/Probe 組合時,先確定 Primer/Probe 想要置放的位置 開啟一個新的 Allelic Discrimination Document, 並把欲設計的序列檔案加入(參照第 23 頁)。

1. 設計 Probe for Allele 1:

在"Sequence" Tab 中,將 SNP 位點 highlight 起來, Edit → Annotate → 按 "SNP Target" இ, 然 後選擇此 SNP 位點的變異型(請參照第 24 頁)。將 Probel 之預定序列 highlight 起來(大約 13~25 bases 之長度)後,先利用 Edit 中 Copy with Allele 1 (Ctrl+Shift+W) 之功能複製序列,再至 Tools→ Primer Probe Test Tool 中,選擇欲設計的 document type (即"TaqMan MGB Allelic Discrimination 或 TaqMan Allelic Discrimination"),並確認 Parameter 設定為"Default",再利用 Paste (Ctrl+V) 將序列貼在 Probel 欄位,此時 Allele 1 variant base 變成小寫的字體 (此例變成"g"),從右邊即可觀察測試序列之 Tm, %GC 和長度是否合適。

註: probe 第一個序列不能為G,且序列裡面C的數目要比G還要多

File		Window Help						
	<u>U</u> ndo	Ctrl+Z) 🕨 🔺 🖷	→ (- 🗉 🤳 🙈 🕂	🕐 🛒 🙉		
	<u>R</u> edo	Ctrl+Y	ion # 3		Primer Prohe Test	Tool		
Se		Ctrl+X	Irder		Parameters			
	Lopy Copy Complement	UIN+C	ก					
1	Copy with Allele 1	Ctrl+Shift+\A(2		Document Type: Tag	1an® MGB Allelic Discriminati 💟 Pa	arameter: Default	Browse
	Copy with Allele 2	Ctrl+Shift+M	∑ 518 to !	541	Primers and Probes			
	Paste	Ctrl+V	·					
AT	<u></u> elete		TGTTCT GCATCTTTAT	(CAAT	Fwd Primer			Im %GC Length
GC	C Select All	Ctrl+A	-IGAAGCC AGAGACCAGA	TTCCT				Tm %GC Length
TC	Annotate	•	AACAGC TCTCAGCCGC	TTATO	Rev Primer			
AT	C Find Sequence	Ctrl+F	AGTCCC AACCTGTGAA	TGTGG				Tm %GC Length
CT	C Find O <u>R</u> F	Ctrl+R	IGGCTGC TCTTCTCCTA	TGGCT	Probe 1	ACGTCTCAGGgTTCGCAGGCAGCT		81.0 63 24
GT	A Find and Exclude		IGGCAGC TCCATGGACG	GGAAG	Probe 2			Tm %GC Length
IT	TGAGGGAA CGTCCCCCA	A CGAGCGCCTT TC	TCCTGATG ATGCCAATTT	TGTGG	TTODE 2			0.0 0 0
GC TG	AICANOCA OCCCAIIOU GCCTAAAC GCCATAACC	n cachaidach ic C agadratraa at	RECOLUCIAR COOODOCICU REGERENT GAGEGETEES	TICCA	Trim			
TT	CCAGTGCA GCGACATGG	G CAGCITCAGC CA	AGGTCTAT GCCTGAGCTG	CAAGA				
CA	GGCAAGAG CAAGAGGCT	C TTECTEATEA EG	CGAGCCCA GTCTCCCTTC	AAAGT	Secondary Structure			
GC	CGGTAGAG CCTACTTTT.	A CCATGTCGCT GC	TGGGAACA AAAGAAGAAA	TAAAG	Oligo	Length	Hairpin Self Dimers Cros	s Dimers
TA	TTCCTTCC TTATCACAC	I GGACAAAGAC AT	CGGCGAGT TGATCCTGCT	CAAGI	Forward Primer	0		
TG	CAGACCAT CATGCTATG	G GGCATAGAAC CT	CACCACTC TGGCCTCATT	CTGAA	🔿 Reverse Primer	0		
TT	GCCCCGAA AATCTGGAT	G ACCTCCAGCT TC.	ACCCGAGC CAGGAGAAAG	TCTTI	O Probe 1	24		
UN	GAIGAGIC ARGAGACCC	A IGUAAAAAAA IA	AAGAAGIC TAIICIII		O Probe 2	0		
					Show 9	Secondary Structure		



此時若 Tm 超過設定值(65℃ to 67℃),可直接在 Probe 1 欄位中框選不同的序列片段,並觀察右邊 對應的 Tm,%GC 和長度,以找出最適當的 Probe (請參照 primer/ probe design guideline)。若框選到 適合的片段,點一下"Trim",就可以將未框選的序列直接刪除,而留下需要的 Probe 序列。

Parameters					
Document Type: T	aqMan® MGB Allelic Discriminati 💌 Parameter: 🛛 Default		🕶 🔳	Browse	
Primers and Probes	3				
		Tm	%GC	Length	
Fwd Prime		0.0	0	0	
DD.i		Tm	%GC	Length	
Hev Prime		0.0	0	0	
Probe 1		Tm	%GC	Length	
110061	<u>Accretes adgrie</u> des ades de la	67.0	57	14	
Probe 2		Tm	%GC	Length	
FIDE 2		0.0	0	0	
Trim					

SNP 位點置放

請將 SNP 位點置於 Probe 序列中間三分之一的區域或從中間到 3'端最後 2 個 base 之前的區域,不 能置放在最後兩個 base,如下圖:





2. 設計 Probe for Allele 2:

在"Sequence" Tab 中,將 Probe2 之預定序列 highlight 起來(大約 13~25 bases 之長度)後,先利用 Edit 中 Copy with Allele 2 (Ctrl+Shift+M) 之功能複製序列,再至 Tools→ Primer Probe Test Tool 中,利用 Paste (Ctrl+V) 將序列貼在 Probe2 欄位,此時 Allele 2 variant base 變成小寫的字體 (此例變成"a"),從右邊即可觀察測試序列之 Tm,%GC 和長度是否合適。

le Eat View Tools Windo	w Help	
Undo Ctri-	•Z 🔰 🕨 🛋 🛤	→ ← Ⅲ 圖 📾 😁 😧 🛒 🙉
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📒 Duệ 🛛 Ctri	•X	
Copy Ctrl-	+C rider	Parametera
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Copy with Allele 1 Ctrl	+Shift+W 522 In	Delever of Decker
Copy with Allele 2 Ctrl	+Shit+M	Primers and Probes
Luu Paste Ctri-	•V futuroutor	Tm %GC Length
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000 Select All Ctri-	*A VSANBOC AGABACCAS	Tm 35C Length
TC# Agnotate	PAACAGC TCTCAGCOG	Rev Pinver 0.0 0 0
ATC End Sequence Ctrl-	+F DISTOCC ANOCTISTON	Tm 35C Length
CTE Find ORF Ctri	*R BECIEC ICTICICCI	Probe 1 ACGTCTCAGGgTTC 67.0 57 14
gts Find and Exclude	SOCAOC TOCATOGAC	Tim 200 Length
TTTGAGOSAA COTCCCCCAA COASC	COCCTT TCTCCTGATG ATSCCAATI	1 PIODe 2 LTLASUBITUSLASULASUL
GCATCAAGCA GCCCATTGCC CACTA	ATGACT TCTACCOCAA 039339CTC	C Tim
TBBOCTAAAC BOCATAAOOC ABAOC	CATCAA ATSTRCCCAT SAGCRCTCC	
TICCASTOCA GOSACATOGS CAGE	TTCAOC CAAOGTCTAT GCCTGAOCT	Secondary Structure
CAGOCAAGAG CAAGAGOCTC TTCCT	TCATCA OGOGAGOCCA GTCTCCCTT	Ofen Leveth Harpin Sel Deves Cross Deves
OCCOSTAGAS CCTACTITIA CCATS	STCOCT OCTOBRANCA AAARAARAA	Cago Lengh
TATTOCTTCC TTATCACACT GOACH	AAAGAC ATCOGCGAGT TGATCCTGC	O Forward Primer 0
TOCAGACCAT CATOCTATOS GOCAL	INSANC CICNCCACIC IOSCOLONI	Reverse Primer 0
THEOCOOSAA AATCIGGATE ACCTO	CCAGCE TCACCOGAGE CAGGAGAAA	OProbe 1 14
CARADADIC AND ACCCA TOCO	ANANA DOUGOUSIC INFICTIT	O Probe 2 20
		Charles Charles
		suow secondadi sancuna

此時若 Tm 超過設定值(65℃ to 67℃),可直接在 Probe 1 欄位中框選不同的序列片段,並觀察右邊 對應的 Tm,%GC 和長度,以找出最適當的 Probe (請參照 primer/ probe design guideline)。若框選到 適合的片段,點一下"Trim",就可以將未框選的序列直接刪除,而留下需要的 Probe 序列。注意 probe1 和 probe2 之間的 Tm 值差異不能超過1℃以上。

Parameters									
Document Type: Taqt	Man® MGB Allelic Discriminati 🝸 Parameter:	Default	(v [8	rowse				
Primers and Probes									
E. J.D.			Tm	%GC	Length				
Fwd Primer			0.0	0	0				
Dav. Diman			Tm	%GC	Length				
Hev Frimer			0.0	0	0				
Dub 1			Tm	%GC	Length				
Probe I	ACGICICAGOGIIC		67.0	57	14				
			Tm	%GC	Length				
Probe 2	СпсАвваттсясАввсАрст		67.0	56	16				
Trim									



3. Forward Primer 設計:

在"Sequence" Tab 中將預定 Primer 序列 highlight 起來(至少 25 bases 之長度,切勿與 Probe 序列重 疊)後,先利用 Edit 中 Copy (Ctrl+C)之功能複製序列,再依下圖至 Tools → Primer Probe Test Tool 中,利用 Paste (Ctrl+V) 將序列貼在 Fwd Primer 欄位,從右邊即可觀察測試序列之 Tm,%GC 和長度是否合適。

🎇 TaqMan® MGB Allelic Discrimination # 3	
Sequence Parameters Primers / Probes Order	
File Name AY228765.txt	
Length 1548 bp. Selection 491 to 513 Double Stranded	_
ATGGGAAATC CCCTCCAAAT CTCCATTTTC CTGGTGTTCT GCATCTTTAT CCAATCAAGT GCTTGTGGAC AAGGCGTGGG AACAGAGCCCC	ITTGGGAGAA 100 🔼
GCCTTGGAGC TACTGAAGCT AGCAAGCCAT TAAAGAAGCC AGAGACCAGA TTCCTGCTCT TCCAAGATGA AAACGATCGC CTGGGCTGTC	STCTCAGACC 200
TCAGCACCCG GAAACACTGC AGGAGTGTGG CTTCAACAGC TCTCAGCCGC TTATCATGAT CATCCACGGG TGGTCGGTGG ATGGCTTGCT	AGAAAACTGG 300
ATCTGGAAGA TAGTGAGTGC GCTGAAGTCC CGACAGTCCC AACCTGTGAA TGTGGGGTTA GTGGACTGGA TCTCCCTGGC ATACCAGCAC	FACACCATTG 400
CTGTTCAAAA CACCCGTATT GTG6GCCAGG ACGTGGCTGC TCTTCTCCTA TG6CTGGAGG AATCT6CGAA GTTTTCTCGG AGCAAAGTTC	ACCTAATTGG 500
GTACAGCCTG GGABCGCACG TCTCAGGTTT CGCAGGCAGC TCCATGGACG GGAAGAACAA GATTGGAAGA ATCACAGGGC TGGACCCTGC	3GGCCCTATG 600
TTTGAGGGAA CGTCCCCCA	
GCATCAAGCA GCCCATTGC	
TGGCCTAAAC GCCATAACC Parameters	
TTCCAGTGCA_GCGACATGC Document Type: TaqMan® MGB Allelic Discriminati 💟 Parameter: Default	Browse
GCCGGTAGAG CCTACTTT, Primers and Prodes	
	Tm %GC Length
IGCAGACCAT CATGCTATU Fwd Primer ACCTAATTGGGTACAGCCTGGGA	61.6 52 23
	Tm %GC Length
CAGATGAGIC AAGAGACCO Rev Primer	0.0 0 0
	Tm %GC Length
Probe 1 ACGTCTCAGGgTTC	67.0 57 14
	Tm %GC Length
Probe 2 TCAGGaTTCGCAGGCA	67.0 56 16
Trim	

確認 Forward Primer 的 Tm 值在 58-60℃,如果 Tm 值不符合,可直接在 Fwd Primer 中框選不同的 序列片段,並觀察右邊對應的 Tm,% GC 和長度,以找出最適當的 Primer (請參照 primer/ probe design guideline),若框選到適合的片段,點一下"Trim",就可以將未框選的序列直接刪除,只留下需要 的序列。

Parameters											
Document Type: Taqt	dan® MGB Allelic Discriminati 💽 Parameter: Default	Browse									
Primers and Probes-	Primers and Probes										
Fwd Primer	ACCTAATTGGGTACAGCCTGGGA	Tm %GC Length 60.1 55 22									
Rev Primer		Tm%GCLength0.000									
Probe 1	ACGTCTCAGGgTTC	Tm %GC Length 67.0 57 14									
Probe 2	TCAGGaTTCGCAGGCA	Tm %GC Length 67.0 56 16									
Trim											



4. Reverse Primer 設計:

在"**Sequence**" Tab 中將預定的 Reverse 序列 highlight 起來(至少 25 bases 之長度, 切勿與 Probe 序列 重疊)後, 然後利用 Edit →" Copy Complement "將序列貼到 Tools → Primer Probe Test Tool 中的 Rev Primer 欄位。

File	Edit View Tools	<u>W</u> indow Help	
	<u>U</u> ndo	Ctrl+Z	
ВШТ-	<u>R</u> edo	Ctrl+Y	ion # 3
	Cu <u>t</u>	Ctrl+X	
Sec	<u>C</u> opy	Ctrl+C	
•	Copy Complement	nt	
	Copy with Allele	Ctrl+Shift	541 to 567 🗖 Double Stranded
	Copy with Allele a	2 Ctrl+Shiπ+M	
لتتنا	Paste	Ctn+v	
ATG	<u>D</u> elete Select All	Ctrl+A	TGTTCT GCATCTTTAT CCAATCAAGT GCTTGTGGAC AAGGCGTGGG AACAGAGGCCC TTTGGGAGAA 100 🔼
GCC -	Appotato	VIII.0	GAAGCC AGAGACCAGA TTCCTGCTCT TCCAAGATGA AAACGATCGC CTGGGCTGTC GTCTCAGACC 200
TCA -	Find Common	CtriteE	AACAGC TCTCAGCCGC TTATCATGAT CATCCACGGG TGGTCGGTGG ATGGCTTGCT AGAAAACTGG 300
ATC	Find OBF	Ctrl+R	AGTCCC AACCTGTGAA TGTGGGGTTA GTGGACTGGA TCTCCCTGGC ATACCAGCAC TACACCATTG 400
CTG	Find and Exclude	5	GGCTGC TCTTCTCCTA TGGCTGGAGG AATCTGCGAA GTTTTCTCGG AGCAAAGTTC ACCTAATTGG 500
GTA_			GGCAGC TCCATGGACG GGAAGAACAA GATTGGAAGA ATCACAGGGC TGGACCCTGC GGGCCCTATG 600
TTTG	AGGGAA CGTCCCCCA		Test Test
TCCAL	CAAGCA GCCCAIIG	ritmer riobe	
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GCCG	GTAGAG CCTACTTT	-Drimoro and Drot	
TATT	CCTTCC TTATCACA	Primers and Proc	
TGCA	GACCAT CATGCTAT		Tm %GC Length
TTGC	CCCGAA AATCTGGA	Fwd Pr	mer ACCIAATTGGGTALAGCCTGGG 60.1 55 22
CAGA	TGAGTC AAGAGACC	D D	Tm %GC Length
		nev Fr	69.7 48 27
		Probe -	1 ACGTCTCAGGoTTC
		1006	67.0 57 14
		Probe	2 TCAGGaTTCGCAGGCA
			67.0 56 16
		Trim	

確認 Reverse Primer 的 Tm 值也能符合在 58-60℃,如果 Tm 值不符合,可直接在 Rev Primer 中框 選不同的序列片段,並觀察右邊對應的 Tm,%GC 和長度,以找出最適當的 Primer (請參照 primer/ probe design guideline),若框選到適合的片段,點一下"Trim",就可以將未框選的序列直接刪除, 只留下需要的序列。

Parameters									
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Primers and Probes									
5 15:		Tn	n %GC	Length					
Fwd Primer	ACCTAATTGGGTACAGCCTGGG	60	.1 55	22					
Dav. Diara		Tn	n %GC	Length					
Hev Primer	IQCAATCITUTTCTTCCCGTCCATGGA	58	8.9 45	22					
Decks 1		Tn	n %GC	Length					
Probe I	ACGICICAGGGIIC	67	.0 57	14					
Decks 0	TEACE-TICCCACCCA	Tn	n %GC	Length					
Probe 2	TLAGGATTLGLAGGLA	67	.0 56	16					
Trim									



決定 primer/ probe set 後可利用 Copy & Paste 功能轉貼到一個新的 text 檔,並 save 起來做為未來參 考資料。另外,也可以把此次設計 document 進行存檔,存檔的方式從 File → Save As 存檔。

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TITGAGOGAA COTCCCCCAA COAGCOCCIT TA		67.0 57 14
GCATCAAGCA GCCCATTGCC CACTATGACT T	Probe 2 TCAGGaTTCGCAGGCA	Tm 23GC Length
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TICCAGIGCA GCGACAIGGG CAGCITCAGC C	Tim	
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CAGATGAGTC AAGAGACCCA TGCAAAAAAA TA	Hairpins not found	
	0 Probe 1 14	
·	O Probe 2 16	
	Show Secondary Structure	

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