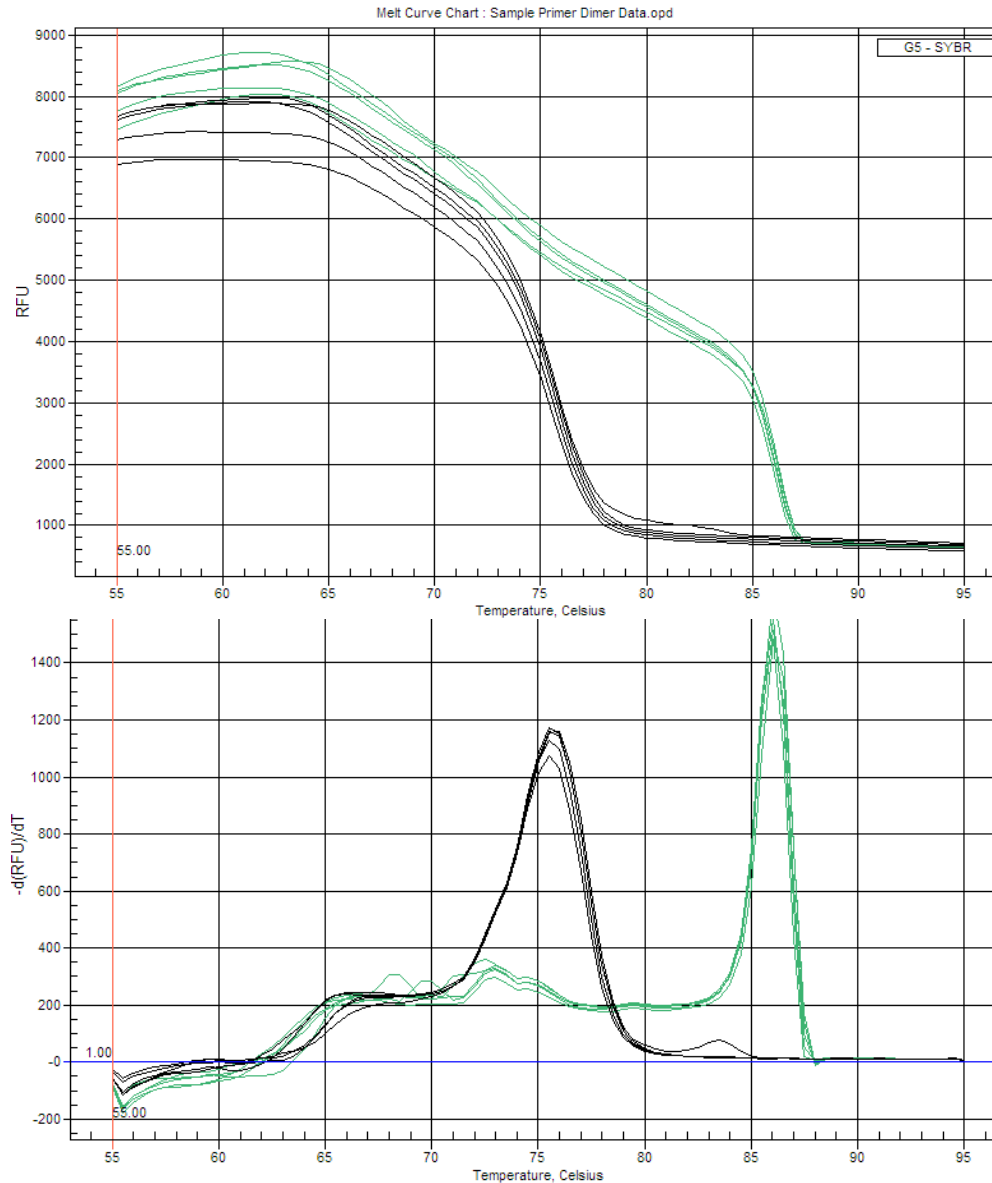


PCR EN TEMPO REAL

MELTING CURVE: CURVA DE DISOCIACIÓN



8/VII/2014

S1401014

APLICACIONES BIOTECNOLOGICAS DE PCR EN TEMPO REAL

DESEÑO DA PCR EN TEMPO REAL

Recopilado por Jorge Rodríguez Castro

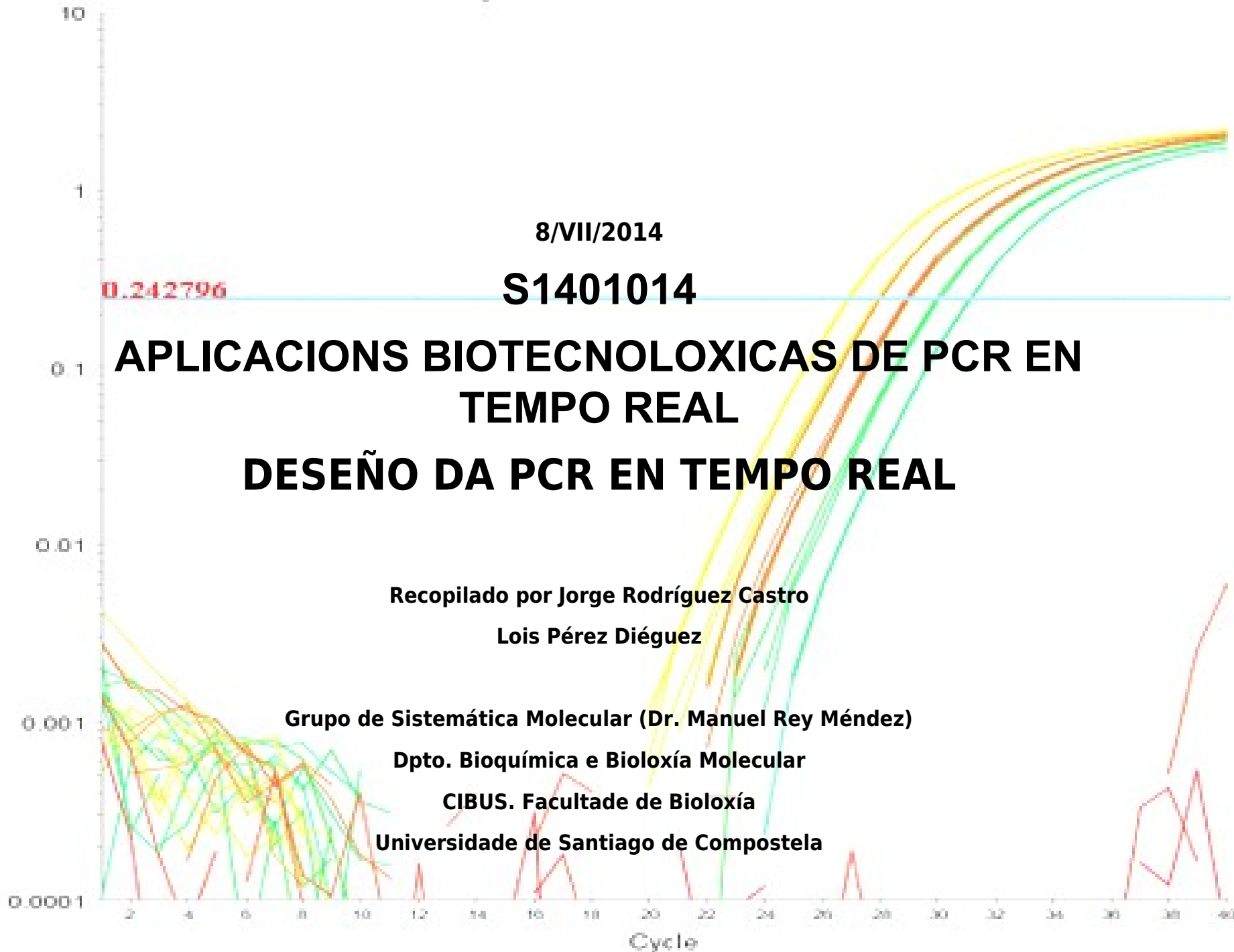
Lois Pérez Diéguez

Grupo de Sistemática Molecular (Dr. Manuel Rey Méndez)

Dpto. Bioquímica e Bioloxía Molecular

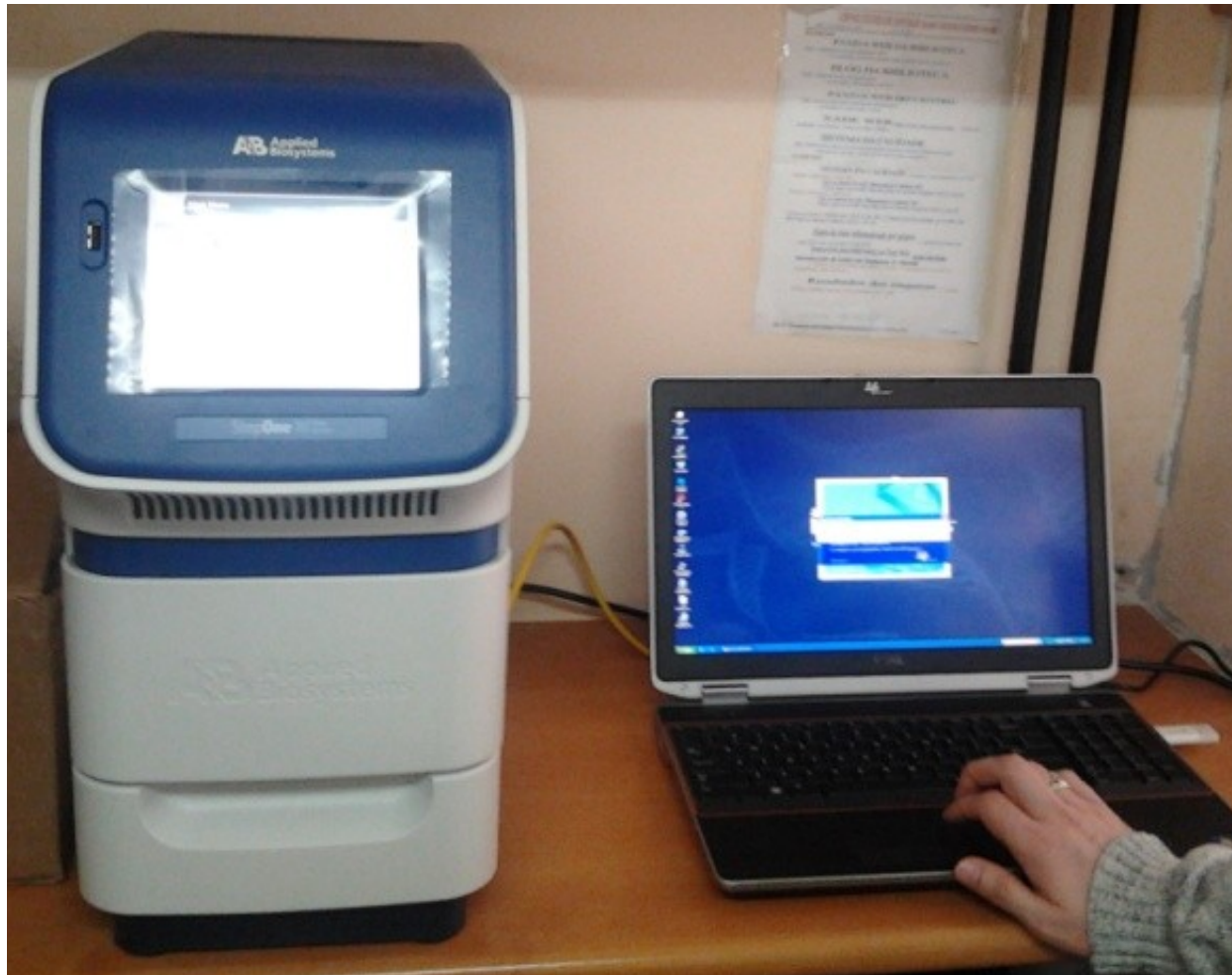
CIBUS. Facultade de Bioloxía

Universidade de Santiago de Compostela



PCR EN TEMPO REAL

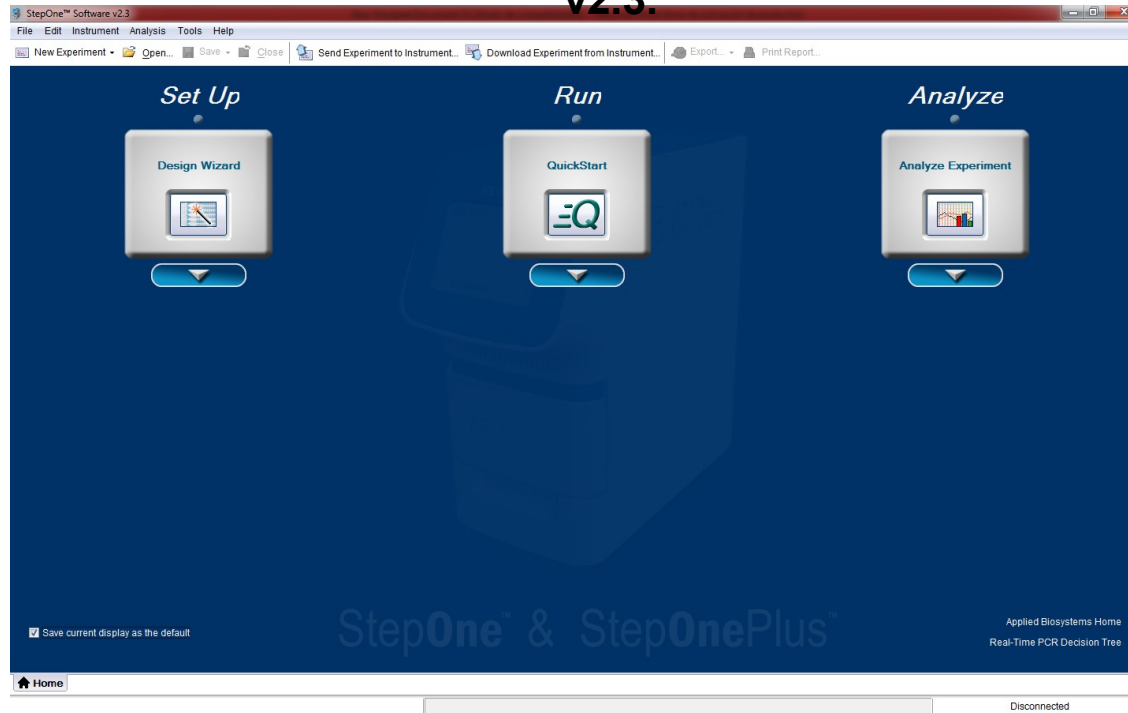
ABI PRISM STEPONE



PCR EN TEMPO REAL

ABI PRISM STEPONE

Entramos no programa **SetpOne Software**
v2.3.



Prememos **SETUP**.

PCR EN TEMPO REAL

ABI PRISM STEPONE: Experiment Properties

Pantalla de Experiment Properties

StepOne™ Software v2.1

File Edit Instrument Analysis Tools Help

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

Experiment: **Untitled** Type: **Quantitation - Standard Curve** Reagents: **TaqMan® Reagents**

Design your experiment...

1. Define

Experiment Properties

2. Set Up

3. Order (optional)

Materials List

1A. Define: Experiment Properties

Instructions: Enter an experiment name, select the instrument type, then select the type of experiment to design.

How do you want to identify this experiment? **Required**

Experiment Name: **Untitled**

Barcode (Optional):

User Name (Optional):

Comments (Optional):

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 3-color, 48-well system.

What type of experiment do you want to design?

Quantitation Genotyping Presence / Absence

Design a gene quantitation experiment to determine the amount of target nucleic acid sequence in a sample.

Previous Finish Designing Experiment Next Cancel

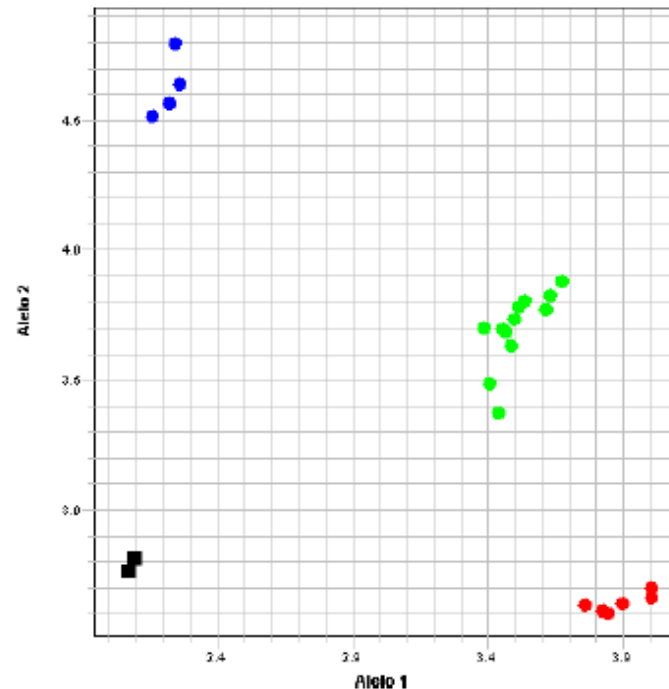
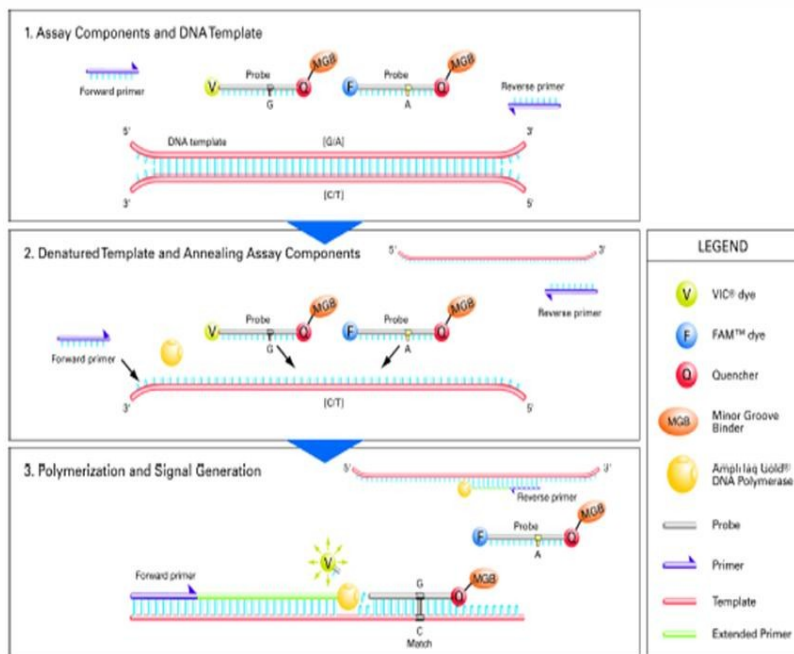
Home Untitled 1 Disconnected

Nomeamos o experimento
Usuario e comentarios.
Marcamos experimento de 48 pocinhos.
Quantification (Genotyping, Presence Absence).
Next

PCR EN TEMPO REAL

ABI PRISM STEPONE: XENOTIPADO

Taqman: Engádese unha sonda complementaria a unha zona interna do fragmento de ADN amplificado. Leva un fluorocromo (emisor) e un “quencher” (captador de fluorescencia), que na etapa de extensión, pola función exonucleasa 5'-3' da polimerasa, se separan e pode detectarse a luz do fluorocromo. Empregando xogos de sondas con fluorocromos de diferentes cores permite estudos de SNPs, diferencias entre secuencias dun só nucleótido. Diferenzar alelos.



PCR EN TEMPO REAL

ABI PRISM STEPONE: Methods & Materials

The screenshot displays the 'StepOne Software v2.3' interface. The main window is titled 'Design your experiment...' and shows the 'Methods & Materials' section for an experiment named 'Practica larvas'. The experiment type is 'Quantitation - Standard Curve' and the reagents are 'SYBR® Green Reagents'. The interface is divided into three main sections: 1. Define, 2. Set Up, and 3. Order (Optional). The '1. Define' section includes 'Experiment Properties', 'Methods & Materials', 'Targets', 'Standards', 'Samples', 'Run Method', and 'Reaction Setup'. The '2. Set Up' section includes 'Targets', 'Standards', 'Samples', 'Run Method', and 'Reaction Setup'. The '3. Order (Optional)' section includes 'Materials List'. The 'Methods & Materials' section contains the following questions and options:

- 1B. Define: Methods & Materials**
Instructions: Select the quantitation method, reagents, ramp speed, and type of template for the real-time PCR reactions.
- Which quantitation method are you using?**
Options: ☒ Standard Curve, ☐ Relative Standard Curve, ☐ Comparative Ct ($\Delta\Delta Ct$)
Note: With the standard curve method, you use standards to determine the absolute quantity of target sequence in a sample.
- Which reagents do you want to use to detect the target sequence?**
Options: ☐ TaqMan® Reagents, ☒ SYBR® Green Reagents
Note: The real-time PCR reactions contain two primers and SYBR® Green I dye. The primers are designed to amplify the target sequence. The SYBR Green dye generates increased fluorescence signal when it binds to double-stranded DNA. Select "Include Melt Curve" to perform melt curve analysis of the amplified sequence.
☒ Include Melt Curve
- Which ramp speed do you want to include in the instrument run?**
Options: ☒ Standard (~ 2 hours to complete a run), ☐ Fast (~ 40 minutes to complete a run)
Note: For optimal results using the standard ramp speed, Applied Biosystems recommends standard reagents for your real-time PCR reactions.
- What type of template do you want to use in the real-time PCR reactions?**
Options: ☐ cDNA (complementary DNA), ☐ RNA, ☒ gDNA (genomic DNA)
Note: You are adding purified gDNA to the real-time PCR reactions. You have already extracted the gDNA from tissue or sample.

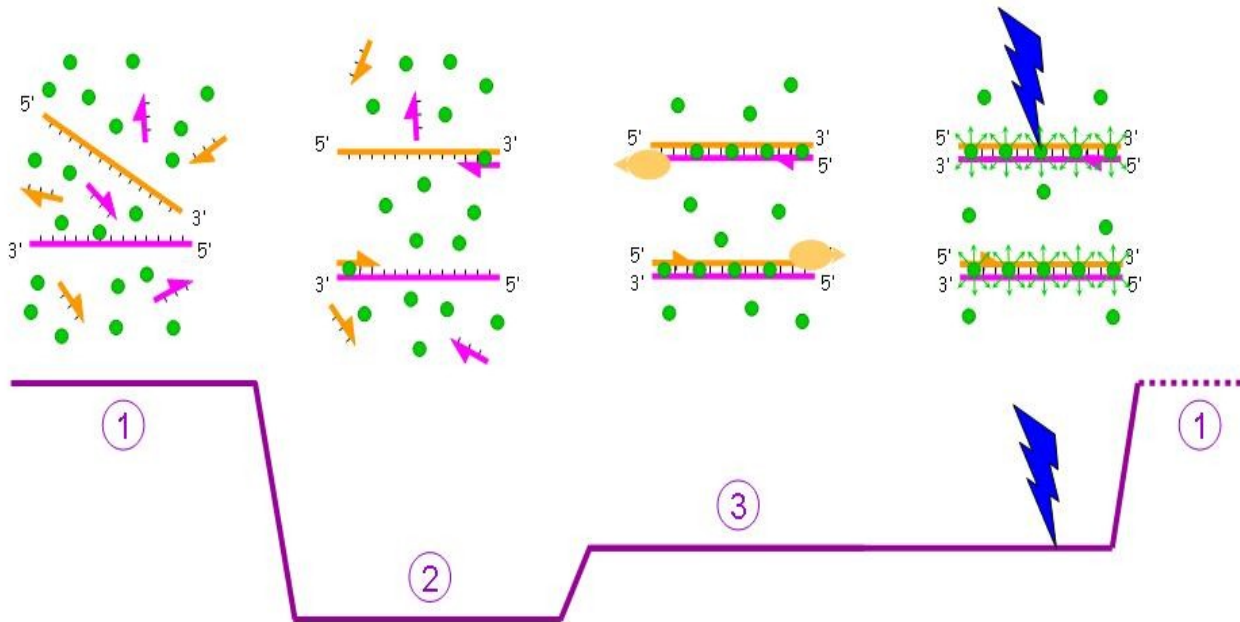
At the bottom of the window, there are navigation buttons: 'Previous', 'Finish Designing Experiment', 'Next', and 'Cancel'. The status bar at the bottom indicates 'Local Instrument: connected'.

Curva Standard, curva estandard relativa, Comparativa de C_T
Reagents. Tipo de química:
SYBR Green, TaqMan

PCR EN TEMPO REAL

ABI PRISM STEPONE

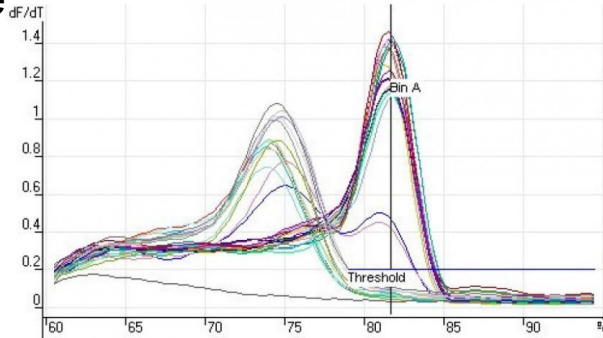
SYBR Green: Sustancia fluorocrómica, que se coloca entre as febras do ADN, producindo maior fluorescencia a maior número de febras de ADN.



PCR EN TEMPO REAL

ABI PRISM STEPONE

Nós imos a empregar o **SYBR Green**, que por defecto marca tamén a **curva de melting** ou fusión. Que vains a indicar se existen amplificacións non buscadas e observar heterocigosidade~



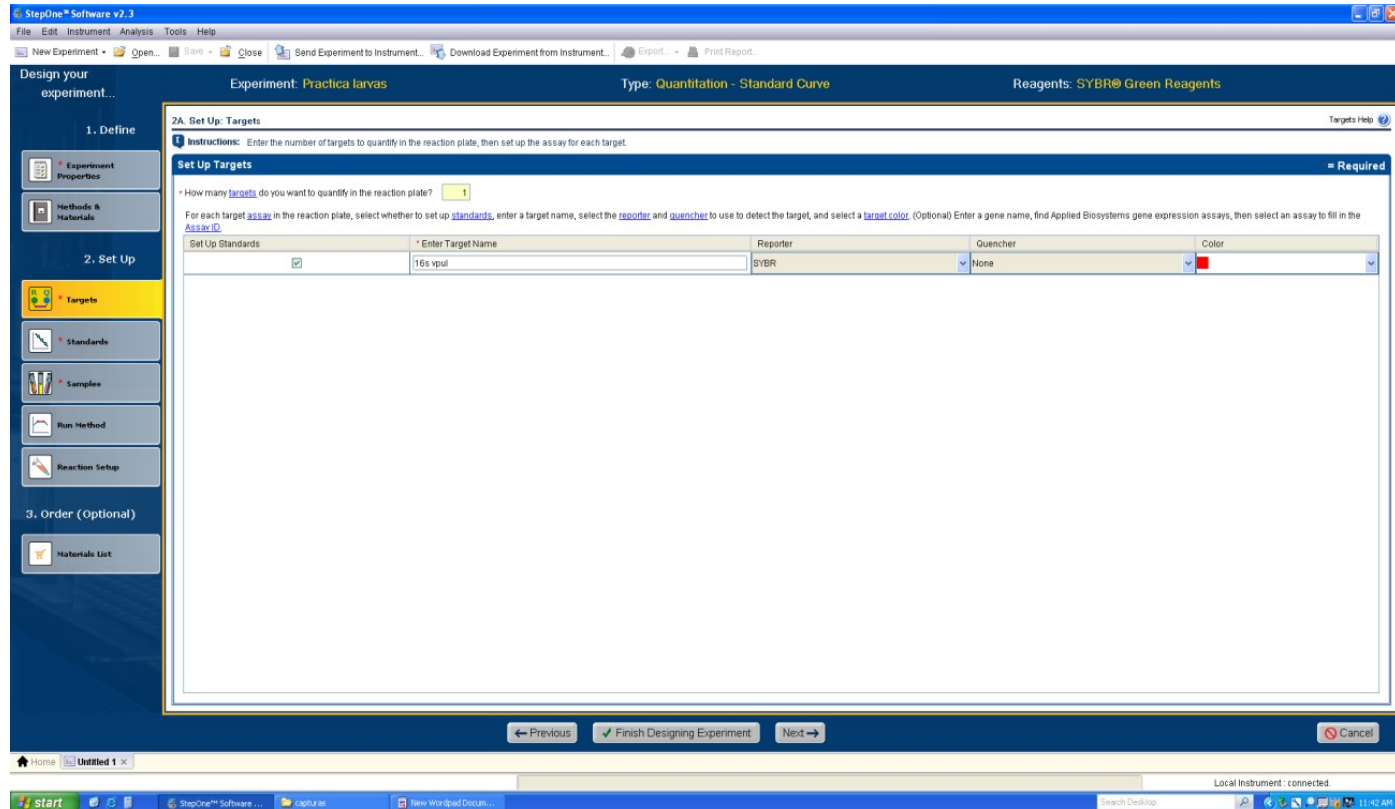
Velocidade das reaccións: **Reacción estándar de 2 horas**, Fast RT de 40'. (precisa reactivos específicos)

Template: cDNA (ADN complementario, a partires de ARN), RNA, **gDNA (ADN xenómico)**

NEXT

PCR EN TEMPO REAL

ABI PRISM STEPONE: SETUP TARGETS



Indicamos o número de mixes distintos (xogos de primers) empregados. O número de dianas (Targets), zonas do ADN que imos analizar.

Método **SYBR**.

Quencher **NONE**. Ningún pois non é un método Taqman.

E asignámoslle unha cor.

NEXT.

PCR EN TEMPO REAL

ABI PRISM STEPONE: SETUP STANDARD

The screenshot displays the StepOne Software v2.3 interface. The main window is titled '2B. Set Up: Standards'. It includes a sidebar on the left with navigation options: '1. Define', '2. Set Up', and '3. Order (Optional)'. The '2. Set Up' section is active, showing 'Targets', 'Standards', 'Samples', 'Run Method', and 'Reaction Setup'. The 'Standards' section is highlighted.

The 'Set Up Standards' panel on the left contains the following information:

- Instructions:** Enter the number of points and replicates for all standard curves in the reaction plate. For each standard curve, enter the starting quantity and select the serial factor.
- Set Up Standards:**
 - How many **points** do you need for each standard curve?
 - How many **replicates** do you need for each point?
 - For each standard curve, define the range of standard quantities by entering the **starting quantity** and **serial factor**.
 - Define the standard curve so that the range of standard quantities spans the expected range of quantities for the unknowns.
 - Table with columns: Target Name, Enter Starting Quantity, Select Serial Factor.
 - Row 1: 16s vpul, 1.0, 2*
- Standard Curve Preview:** A graph showing a standard curve for 16s vpul. The y-axis is labeled with values: 3.2E1, 1.6E1, 8E0, 4E0, 2E0, 1E0. The x-axis represents the serial factor.
- Well Count:** 6 - Unknown, 30 - Standard, 3 - Negative Control, 9 - Empty.

The 'View Plate Layout' panel on the right shows a 96-well plate layout. It includes options to 'Arrange Plate by: Columns' and 'Place Negative Controls in: Lower Right'. The plate layout is displayed as a grid with columns 1-8 and rows A-F. The layout shows the placement of standards, samples, and negative controls.

Puntos da recta patrón. Polo xeral 5 ou 6

Número de replicas para cada punto

Concentración inicial

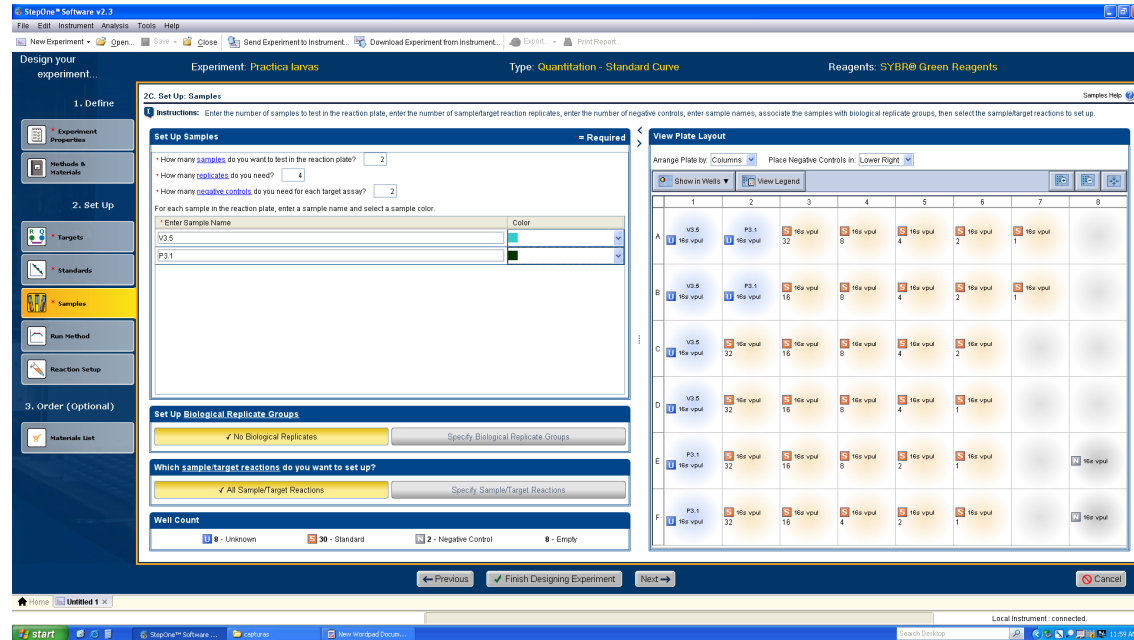
Factor de dilución x2

Organización das mostras e controis negativos na placa: Filas ou por columnas.

Next

PCR EN TEMPO REAL

ABI PRISM STEPONE: SETUP SAMPLES



Número de muestras de ADN
Número de replicas por muestras
Número de controles negativos.

Cores das muestras

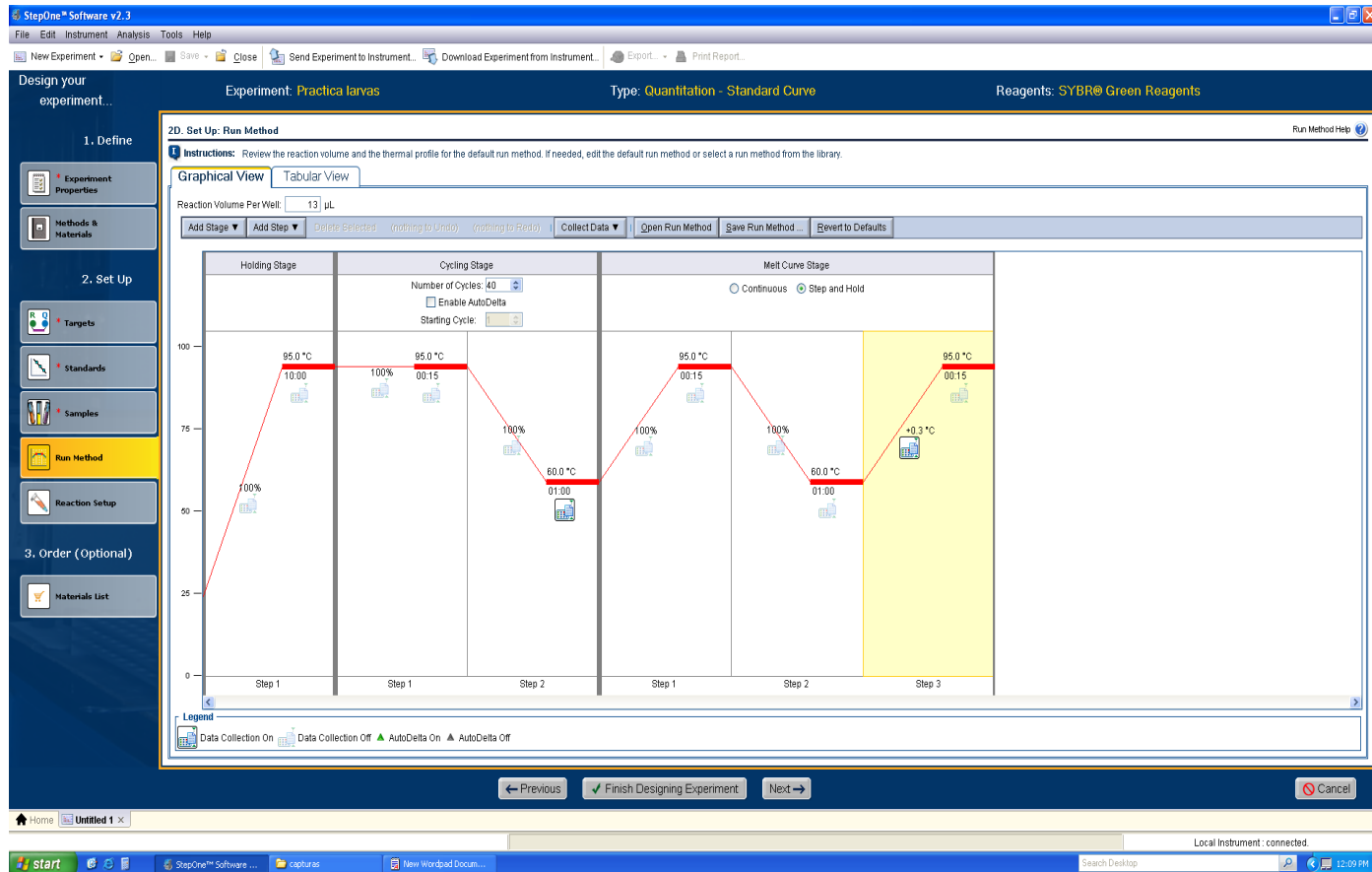
No Biological Replicates (só unha extracción de cada ADN)

Imos cargar na placa todas as muestras con tódolos xogos de primers: **All Sample.**

Next

PCR EN TEMPO REAL

ABI PRISM STEPONE: RUN METHOD



Volume final dos pociños.
Número de ciclos.
Next

PCR EN TEMPO REAL

ABI PRISM STEPONE: REACTION SETUP

StepOne Software v2.3

File Edit Instrument Analysis Tools Help

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

Design your experiment... Experiment: **Practica larvas** Type: **Quantitation - Standard Curve** Reagents: **SYBR® Green Reagents**

1. Define

2. Set Up

3. Order (Optional)

Materials List

2F. Set Up: Reaction Setup

Instructions: For each target assay in the reaction plate, select the assay type (if using TaqMan reagents), then review the calculated volumes for preparing the standard dilution series, samples, and PCR reactions. If needed, edit the reaction volume, excess reaction volume, component concentrations, and/or stock concentrations. Click "Print Reaction Setup" to print instructions on how to prepare the PCR reactions.

Select Tab: **Reaction Mix Calculations** Sample Dilution Calculations

Reaction Volume Per Well: 13.0 µL Excess Reaction Volume: 10 %

Master Mix Concentration: 2.0 x

Forward Primer Starting Concentration: 20.0 pmol/µL Forward Primer Final Concentration: 300.0 nM

Reverse Primer Starting Concentration: 20.0 pmol/µL Reverse Primer Final Concentration: 300.0 nM

Component	Volume (µL) for 1 Reaction
Master Mix (2.0x)	6.50
Forward Primer	0.20
Reverse Primer	0.20
Template (1.0x or Standard)	1.30
H ₂ O	4.81
Total Volume	13.00

Standard Dilution Series for 16x vpu

Dilution Point	Source	Source Volume (µL)	Diluent Volume (µL)	Total Volume (µL)	Standard Concentration (ng/µL)
1 (C2)	Stock	3.87	11.86	15.73	24.6
2 (F8)	Dilution 1	7.86	7.86	15.73	12.3
3 (F8)	Dilution 2	7.86	7.86	15.73	6.2
4 (F8)	Dilution 3	7.86	7.86	15.73	3.1
5 (C2)	Dilution 4	7.86	7.86	15.73	1.6
6 (C1)	Dilution 5	7.86	7.86	15.73	0.8

Standard Concentration in Stock: 100.0 ng µL

Standard Concentration in Stock: 100.0 ng µL

Previous Finish Designing Experiment Next

Cancel

Home Untitled 1

Local Instrument: connected

Search For: []

12:40 PM

Cálculos das cantidades de cada un dos reactivos

Exceso de MIX: 10%, necesario para compensar os “erros” de pipeteo.

Master Mix posúe unha concentración inicial de 2x, polo que no volúmen final queda diluído a metade.

Concentración inicial dos primers.

Concentración final dos primers (50 a 900nM)

A cantidade de ADN aparece fixada nun 10% do volume final

Next

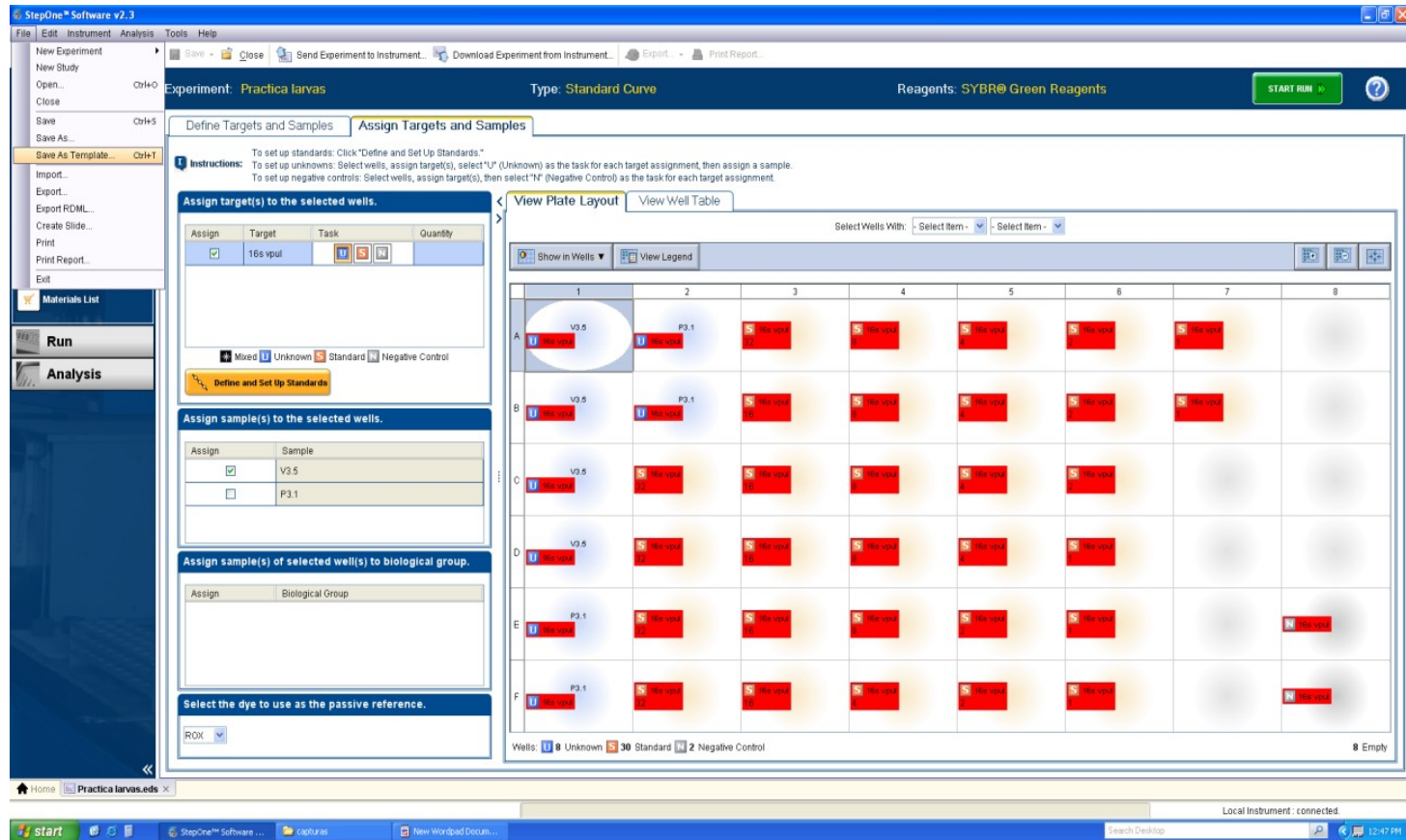
PCR EN TEMPO REAL

ABI PRISM STEPONE: MATERIALS LIST

Para mercar os reactivos necesarios de BIOSYSTEM vía Internet.

PCR EN TEMPO REAL

ABI PRISM STEPONE



Antes de comenzar a PCR picando en **RUN** (parte superior derecha en verde) podemos editar o deseño da placa modificando a posición e cores das dianas, mostrars e controis negativos. Podemos salvar o arquivo e o deseño da placa para outras reaccións.

RUN START

PCR EN TEMPO REAL

PROTOCOLO

- 1) Deseño de dúas PCRs para a detección de ADN de Cabalo e Vaca, con dúas réplicas e un control negativo para cada especie (tres tubos cun volume de 25ul para cada especie):

Reactivo	Conc. inicial	Conc. final	25 µl	x 3 tubos
SYBR Green Master mix 2X	2X	1X	12,5	37,5
Cebador F	20 µM	300 nM	0,375	1,125
Cebador R	20 µM	900 nM	1,125	3,375
ADN			(2)	(6)
H ₂ O desionizada autoclavada	-	-	9	27

- 2) Identificar un tubo de 1.5 ml como MIX Cabalo e outro como MIX Vaca.
- 3) Engadirilles a cada un **27 µl** de **auga** desionizada autoclavada.
- 4) Engadirilles **37,5 µl** de **SYBR Green Master mix 2X**.
- 5) Engadirilles **1,125 µl** do **Cebador F** correspondente.
- 6) Engadirilles **3,375 µl** do **Cebador R** correspondente.
- 7) Mezclade cada tubo cun vortex (pode ser necesario centrifugalo despois para baixar as gotas das paredes) ou pipeteando.

PCR EN TEMPO REAL

PROTOCOLO

- 8) Coller seis tubos de 200 µl e identificalos C1, C2, C3, V4, V5, V6 e o código da vosa mostra.
- 9) Pipetear en tres deles **23 µl** do MIX Cabalo e nos outros tres, **23 ul** do MIX vaca
- 10) Pipetear 2 µl de ADN nos tubos 1 e 2 de cada especie (os tubos 3 son os controis negativos).

C1	C2	C3	V4	V5	V6
2 µl	2 µl	C-	2 µl	2 µl	C-

PCR EN TEMPO REAL

PREPARACIÓN DA RECTA PATRÓN

- 11) Os grupos 1 e 2 farán a preparación da recta patrón de Cabalo e os grupos 3 e 4 as de Vaca.
- 12) Cada grupo colle o ADN Patrón que lle corresponda (Grupos 1 e 2 o ADN de Cabalo e Grupos 3 e 4 o de Vaca) e fará 3 dilucións seriadas en tres tubos de 1,5 ml.

1/1	1/10	1/100	1/1000
Patrón	2 ul Patrón +18 ul H ₂ O	2 ul 1/10 +18 ul H ₂ O	2 ul 1/100 +18 ul H ₂ O

Reactivo	Conc. inicial	Conc. final	25 µl	x 5 tubos
SYBR Green Master mix 2X	2X	1X	12,5	62,5
Cebador F	20 µM	300 nM	0,375	1,875
Cebador R	20 µM	900 nM	1,125	5,625
ADN			(2)	(10)
H ₂ O desionizada autoclavada	.	.	9	45

PCR EN TEMPO REAL

PREPARACIÓN DA RECTA PATRÓN

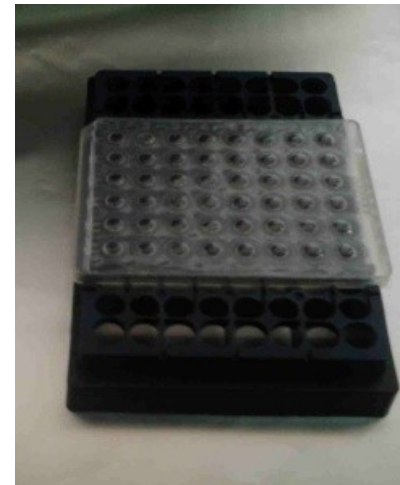
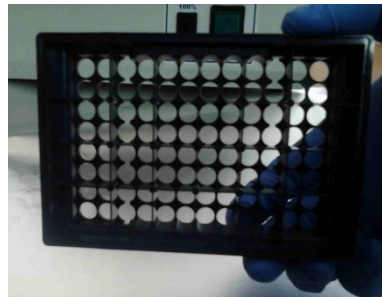
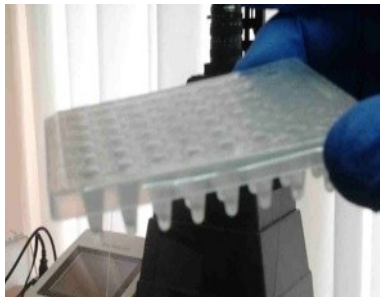
- 13) Identificar un tubo de 1.5 ml como MIX Cabalo e outro como MIX Vaca.
- 14) Engadirilles a cada un **45** µl de auga desionizada autoclavada.
- 15) Engadirilles **62,5** µl de SYBR Green Master mix 2X.
- 16) Engadirilles **1,875** µl do Cebador **F correspondente a cada grupo**.
- 17) Engadirilles **5,625** µl de Cebador **R correspondente a cada grupo**.
- 18) Mezclade cada tubo cun vortex (*pode ser necesario centrifugalo despois para baixar as gotas das paredes*) ou pipeteando.
- 19) Coller cinco tubos de 200 µl e identificalos 1/1, 1/10, 1/100, 1/1000, C- e o código do voso patrón (C ou V).
- 20) Pipetear en cada un deles **23** µl do MIX.
- 21) Pipetear 2 µl de cada dilución do Patrón que lle corresponda (excepto no primeiro que vai o patrón sen diluir).

1/1	1/10	1/100	1/1000	C-
2 µl do Patrón sen diluir	2 µl	2 µl	2 µl	-

PCR EN TEMPO REAL

PREPARACIÓN DA PLACA

- 22) O grupo 5 pasa o contido dos tubos á placa de 48 posta no soporte para evitar que se manche a parte inferior..

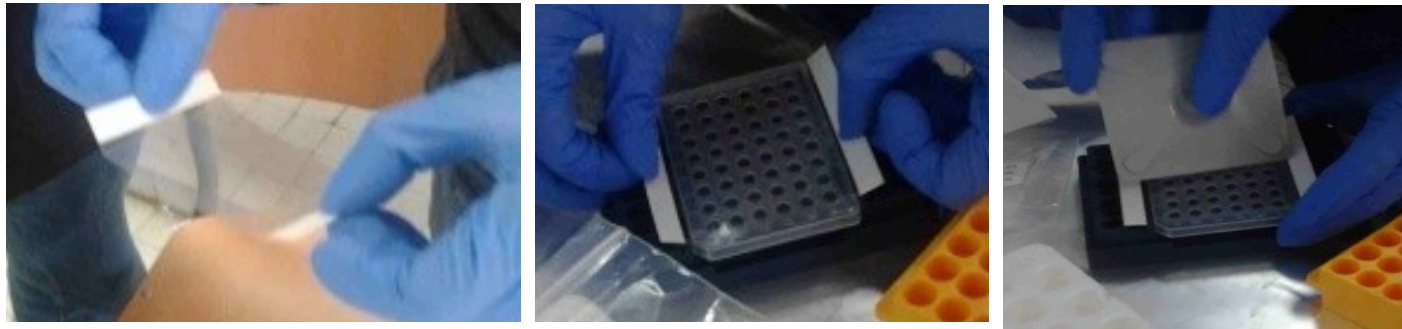


	1	2	3	4	5	6	7	8
A	Grupo1 Cabalo	Grupo1 Cabalo	Grupo1 C-	Grupo1 Vaca	Grupo1 Vaca	Grupo1 C-	Patrón 1/1 Vaca	Patrón 1/1 Vaca
B	Grupo2 Cabalo	Grupo2 Cabalo	Grupo2 C-	Grupo2 Vaca	Grupo2 Vaca	Grupo2 C-	Patrón 1/10 Vaca	Patrón 1/10 Vaca
C	Grupo3 Cabalo	Grupo3 Cabalo	Grupo3 C-	Grupo3 Vaca	Grupo3 Vaca	Grupo3 C-	Patrón 1/100 Vaca	Patrón 1/100 Vaca
D	Grupo4 Cabalo	Grupo4 Cabalo	Grupo4 C-	Grupo4 Vaca	Grupo4 Vaca	Grupo4 C-	Patrón 1/1000 Vaca	Patrón 1/1000 Vaca
E	Grupo5 Cabalo	Grupo5 Cabalo	Grupo5 C-	Grupo5 Vaca	Grupo5 Vaca	Grupo5 C-		
F	Patrón 1/1 Cabalo	Patrón 1/1 Cabalo	Patrón 1/10 Cabalo	Patrón 1/10 Cabalo	Patrón 1/100 Cabalo	Patrón 1/100 Cabalo	Patrón 1/1000 Cabalo	Patrón 1/1000 Cabalo

PCR EN TEMPO REAL

PREPARACIÓN DA PLACA

- 23) Selar a placa coa tapa adhesiva transparente, tendo coidado de tocar só no borde da mesma.



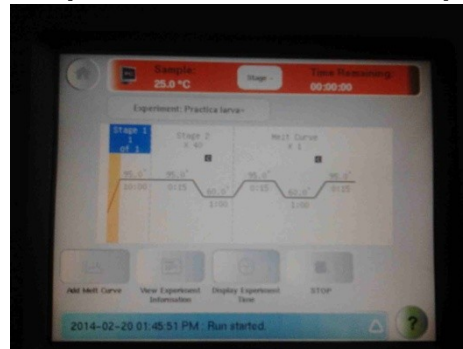
- 24) Centrifugar a placa se non está todo o líquido no fondo.
- 25) Programar o termociclador de tempo real e meter a placa.

Ciclo de PCR:

95° C-10', (95° C-15'', 60° C-1')x40,

Curva de disociación

95° C-15'', 60° C-1', 95° C-15''



PCR EN TEMPO REAL

RUN

TapMan™ Software v2.3

File Edit Instrument Analysis Tools Help

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

Experiment Menu Experiment: **Practica larvas** Type: **Standard Curve** Reagents: **SYBR® Green Reagents** **START RUN**

Setup
Experiment Properties
Plate Setup
Run Method
Reaction Setup
Materials List
Run
Analysis

Reaction Setup

Instructions: For each target assay in the reaction plate, select the assay type (if using TapMan reagents), then review the calculated volumes for preparing the standard dilution series, samples, and real-time PCR reactions. If needed, edit the reaction volume, excess reaction volume, component concentrations, and/or stock concentrations. Click "Print Reaction Setup" to print instructions on how to prepare the PCR reactions.

Reaction Mix Calculations **Sample Dilution Calculations**

Select Tab: **Reaction Mix Calculations** Reaction Volume Per Well: 12 µL Excess Reaction Volume: 10 % **Print Reaction Setup**

Reactions for 16s vpu

Master Mix Concentration: 2.0 x
Forward Primer Starting Concentration: 20.0 pmol/µL Forward Primer Final Concentration: 300.0 nM
Reverse Primer Starting Concentration: 20.0 pmol/µL Reverse Primer Final Concentration: 300.0 nM

Component	Volume (µL) for 1 Reaction
Master Mix (2.00)	6.90
Forward Primer	0.18
Reverse Primer	0.18
Sample (1.00) or Standard	1.20
H ₂ O	4.44
Total Volume	12.00

Standard Dilution Series for 16s vpu

Dilution Point	Source	Source Volume (µL)	Diluent Volume (µL)	Total Volume (µL)	Standard Concentration (ng/µL)
1 (32)	Stock	3.87	18.65	14.52	26.7
2 (16)	Dilution 1	7.26	7.26	14.52	13.3
3 (8)	Dilution 2	7.26	7.26	14.52	6.7
4 (4)	Dilution 3	7.26	7.26	14.52	3.3
5 (2)	Dilution 4	7.26	7.26	14.52	1.7
6 (1)	Dilution 5	7.26	7.26	14.52	0.8

Starting run
Starting the instrument run...

Local Instrument: connected

PCR EN TEMPO REAL

RUN

StepOne™ Software v2.3

File Edit Instrument Analysis Tools Help

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

Experiment Menu Experiment: **Practica larvas** Type: **Standard Curve** Reagents: **SYBR® Green Reagents** **START RUN**

Setup

- Experiment Properties
- Plate Setup
- Run Method
- Reaction Setup**
- Materials List

Run

Analysis

Reaction Setup

Instructions: For each target assay in the reaction plate, select the assay type (if using TaqMan reagents), then review the calculated volumes for preparing the standard dilution series, samples, and real-time PCR reactions. If needed, edit the reaction volume, excess reaction volume, component concentrations, and/or stock concentrations. Click "Print Reaction Setup" to print instructions on how to prepare the PCR reactions.

Reaction Mix Calculations **Sample Dilution Calculations**

Select Target: **16s vpul**

Reaction Volume Per Well: **12** μ L Excess Reaction Volume: **10** %

Reactions for 16s vpul

Master Mix Concentration: **2.0** X

Forward Primer Starting Concentration: **20.0** pmol/ μ L Forward Primer Final Concentration: **300.0** nM

Reverse Primer Starting Concentration: **20.0** pmol/ μ L Reverse Primer Final Concentration: **300.0** nM

Component	Volume (μ L) for 1 Reaction
Master Mix (2.0X)	6.00
Forward Primer	0.18
Reverse Primer	0.18
Sample (10X) or Standard	1.20
H ₂ O	4.44
Total Volume	12.00

Starting run

Starting the instrument run...

Standard Dilution Series for 16s vpul

Standard Concentration in Stock: **100.0** ng per μ L

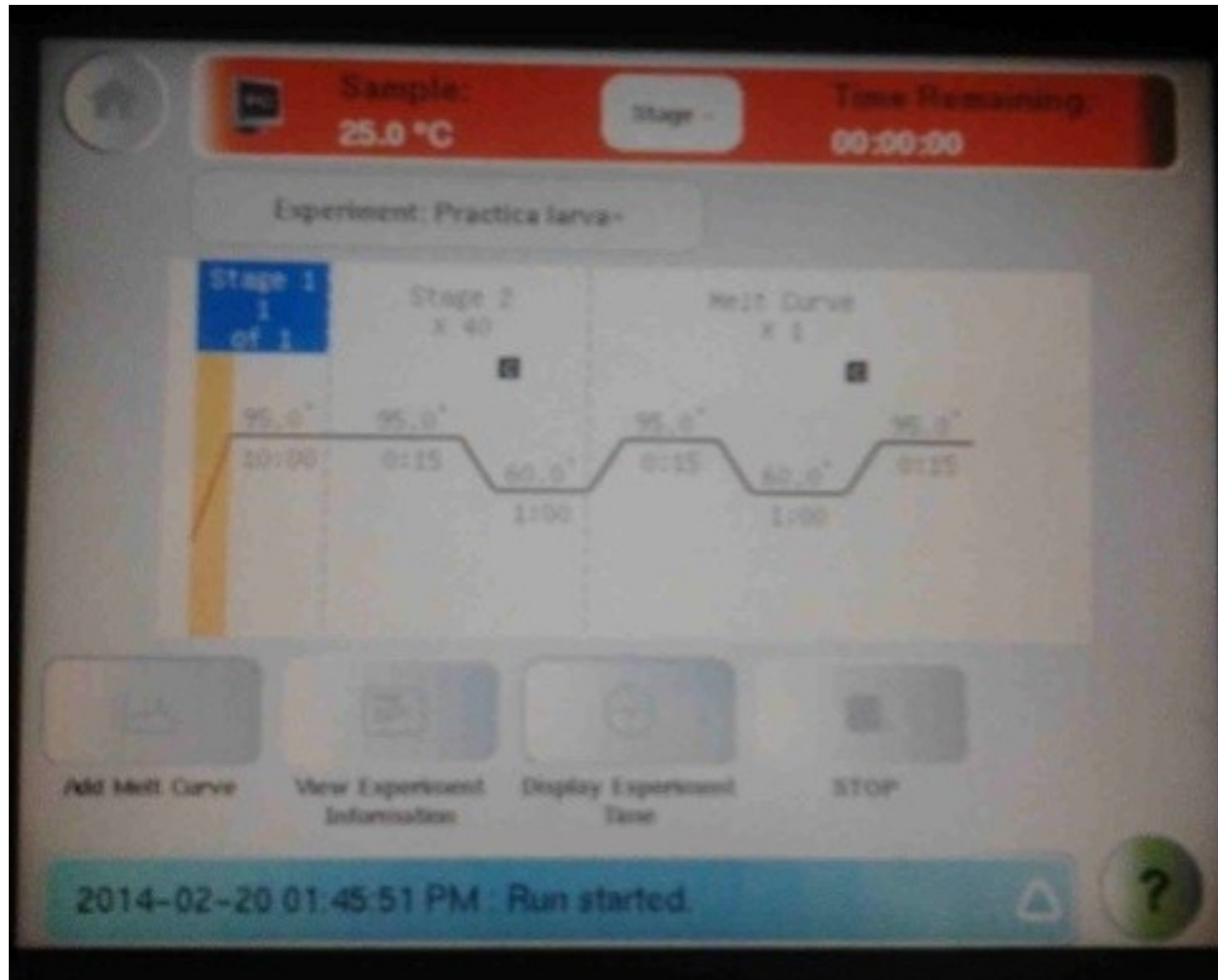
Dilution Point	Source	Source Volume (μ L)	Diluent Volume (μ L)	Total Volume (μ L)	Standard Concentration (ng/ μ L)
1 (32)	Stock	3.87	10.65	14.52	26.7
2 (16)	Dilution 1	7.26	7.26	14.52	13.3
3 (8)	Dilution 2	7.26	7.26	14.52	6.7
4 (4)	Dilution 3	7.26	7.26	14.52	3.3
5 (2)	Dilution 4	7.26	7.26	14.52	1.7
6 (1)	Dilution 5	7.26	7.26	14.52	0.8

Local Instrument: connected.

1:45 PM

PCR EN TEMPO REAL

RUN



PCR EN TEMPO REAL

RUN

