PCR method course

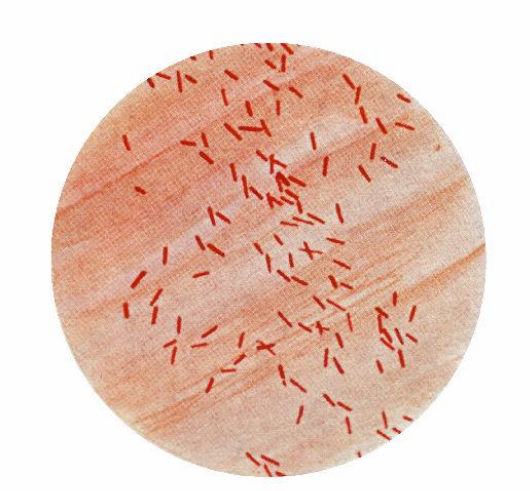
9-11, Nov., 2013 at NIN

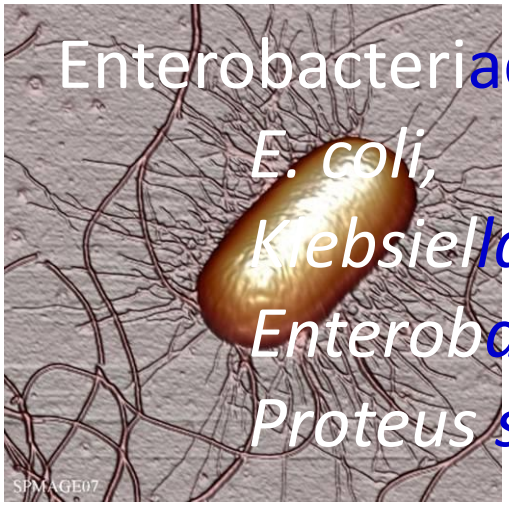
Teacher: Ph.D Atsushi HINENOYA (OPU)
Group 1: Mr. Nguyen Do Phuc
Mr. Le Quoc Phong
Ms. Tran Thi My Duyen
Ms. Bui Thi Kim Ngan

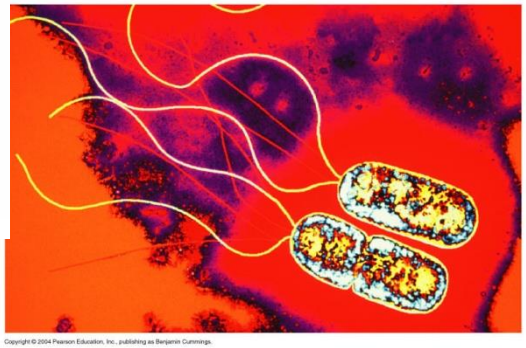
GROUP 1

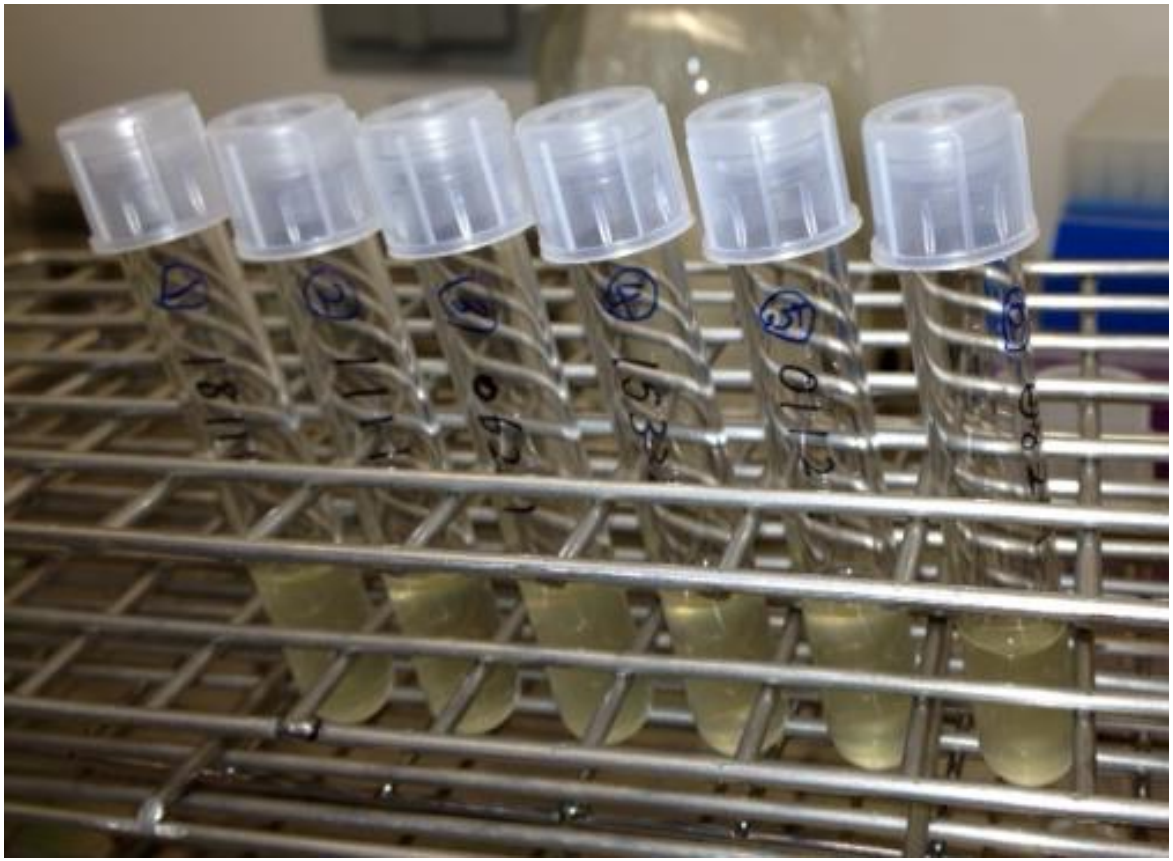
Code of samples

- **1:** 18111
- **2:** 11111
- **3:** 06221
- **4:** 15322
- **5:** 01122
- **6:** 08211









1: 18111: K.pneuminiae CTX-M1, SHV
2: 11111: E.coli CTX-M1; CTX-M4; TEM
3: 06221 : E.coli CTX-M1; CTX-M2; CTX-M3; CTX-M4; SHV; TEM
4: 15322: E.coli ESBL
5: 01122: E.coli CTX-M4 group
6: 08211: E.coli CTX-M1

Prepare reagents

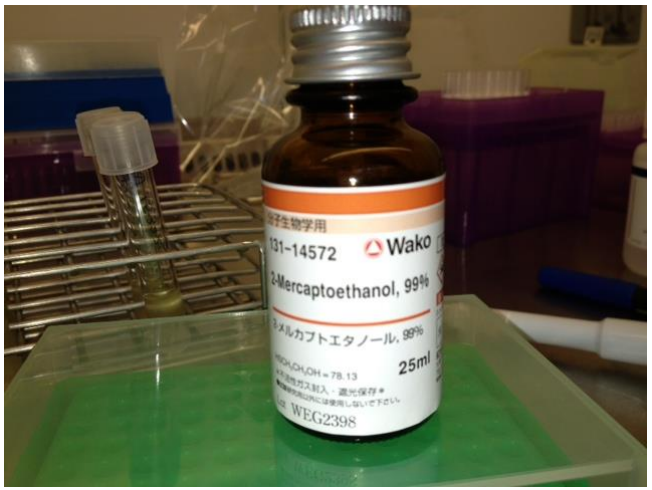
- **TE buffer containing 1mg/ml lysozyme**:

- 7mg lysozyme + 700ml TE (10X solution)

- Dilute: 1 X: 1µl (10X) + 9 1µl TE

- **buffer RLT** :

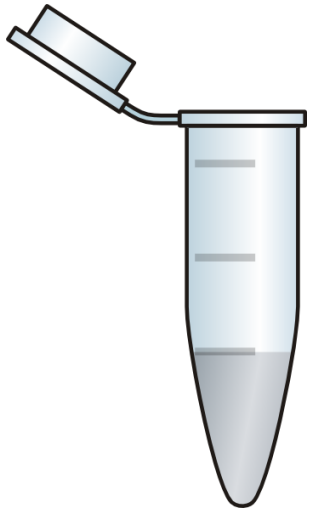
- 50µl β-mercaptoethanol + 5ml buffer RLT Lysis



RNA isolation

- **Procedure:**

1- 500 µl (bacterial culture)



Centrifuge 5000 *g*/10 min

Decant supernatant

2- Add 200 µl TE buffer containing 1mg/ml lysozyme : Mix by vortex/10s, RT/10 min

3- Add 700 µl buffer RLT : Mix by vortex

4- Add 500 µl ethanol 96-100% : Mix by pipetting (bacterial lysate)

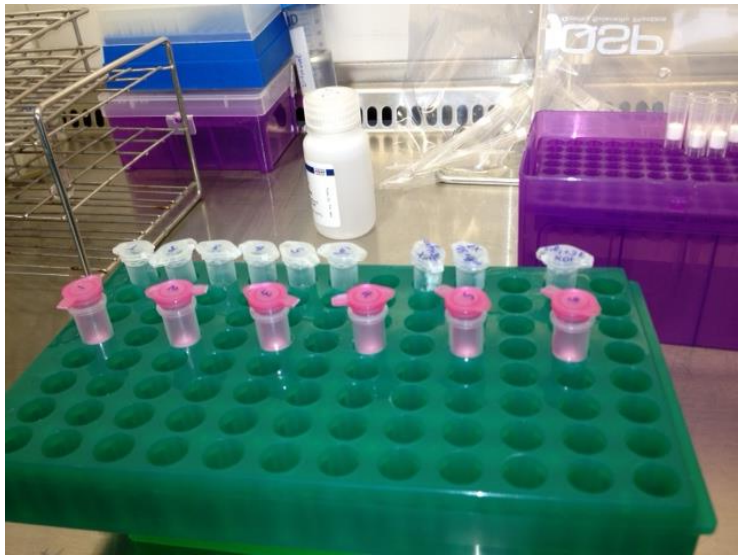
Purification RNA from bacterial lysate Using Rneasy Mini Kit

- **Procedure:**

1- Add 500 µl lysate to Mini spin Column, centrifuge 8.000 *g*/15 s to discard flow through (2 times)

2- Add 700 µl RW1 to Mini spin Column, centrifuge 8.000 *g*/15 s to wash spin column membrane

3- Place Mini spin Column in new tube. Add 500 µl RPE to Mini spin Column, centrifuge 8.000 *g*/15 s to wash spin column membrane



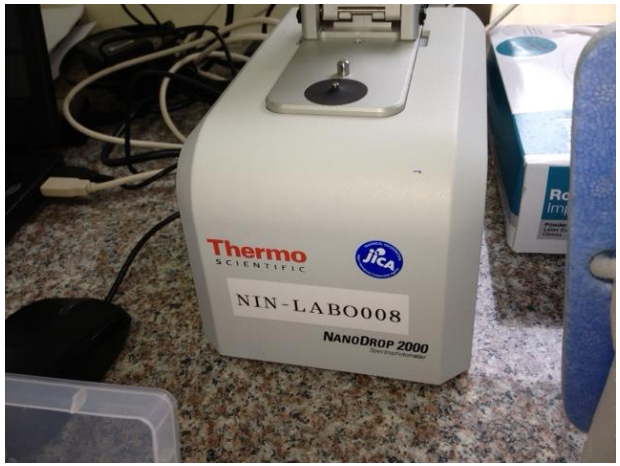
Purification RNA from bacterial lysate Using Rneasy Mini Kit (cont.)

- **Procedure:**

4- Add 500 µl RPE to Mini spin Column, centrifuge 8.000 *g*/2 min to wash spin column membrane to ensure that no ethanol is carried over during elution stage.

5- Place Mini spin Column in new tube. Add 30µl Re-free water, centrifuge 8.000 *g*/1 min

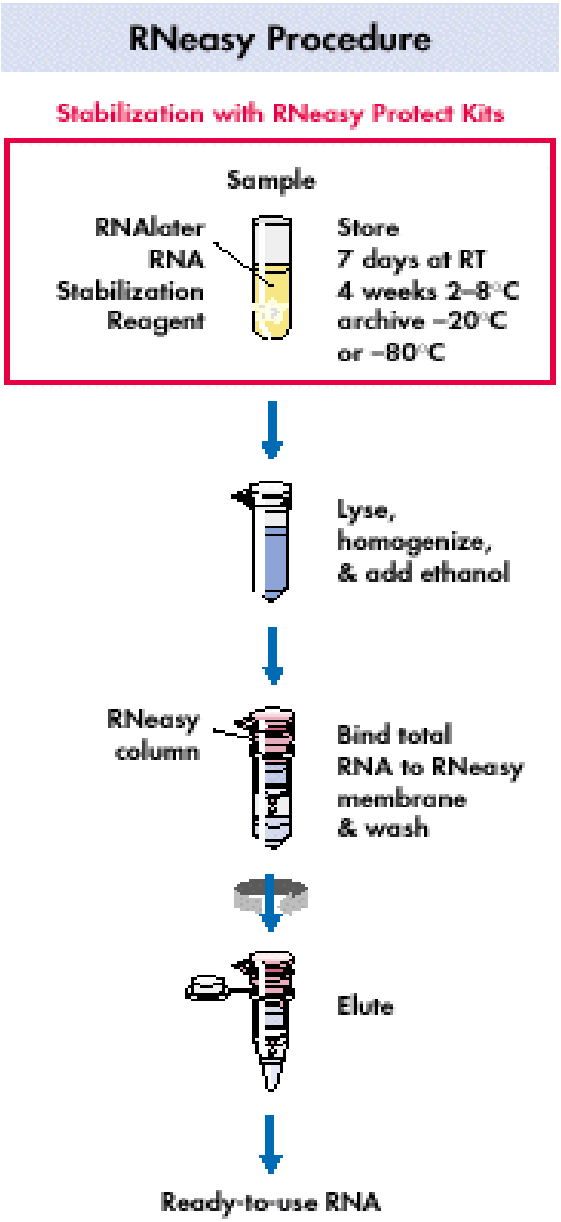
6- Measure RNA concentration:




QIAGEN
RNeasy® Mini Kit (50)
Cat. No. 74104
Store at room temperature (15–25°C)
For molecular biology applications
Not intended for diagnostic purposes
Lot No. 145035572
0074104000000145035572
QIAGEN
研究用試薬
保存条件　室温（15～25℃）


RNeasy® MinElute™
Cleanup Kit (50)
QIAGEN

Flow chart of RNeasy Protect Mini Kit



RNA concentration

First elution (ng/µl)		second elution(ng/µl)	
1-1:	20.3	1-2:	5.5
2-1:	19.3	2-2:	12.4
3-1:	36.7	3-2:	22.4
4-1:	32.8	4-2:	12.3
5-1:	12.2	5-2:	7.3
6-1:	12.6	6-2:	6.3

Prepare cDNA (Takara kit for research)

1. Genomic DNA elimination:

• Reagent	one reation	6 reactions
- 5X gDNA Eraser buffer	2 µl	12 µl
- gDNA Eraser	1 µl	6 µl
- Rnase free water	2 µl	12 µl

- For each reaction for Total RNA : 5 µl

42°C, 2 min (Reaction solution)

Keep 4°C

Prepare cDNA (cont.)

2. Reverse-transcription reaction:

Code of samples:

With RT enzyme Mix 1	Without RT enzyme Mix 1
- Duyen: 1, 2, 3	1, 2, 3
- Phuc: 4, 5, 6	4, 5, 6
- Phong: 1, 2, 3	1, 2, 3
- Ngan: 4, 5, 6	4, 5, 6

Prepare cDNA (cont.)

- **Reverse-transcription reaction (SYBR Green qPCR)**

Reagent	1 reation	6 (with En)	6 (Without En)
- Reaction solution	10 µl	/	/
- 5X PrimerScrip Buffer 2	4 µl	24 µl	24 µl
- PrimerScrip RT Enzyme Mix1	1 µl	6 µl	no
- RT Primer Mix	1 µl	6 µl	6 µl
- Rnase free water	4 µl	24 µl	24 µl

- For each reaction for Total Reaction solution : 10 µl

  37°C, 15 min

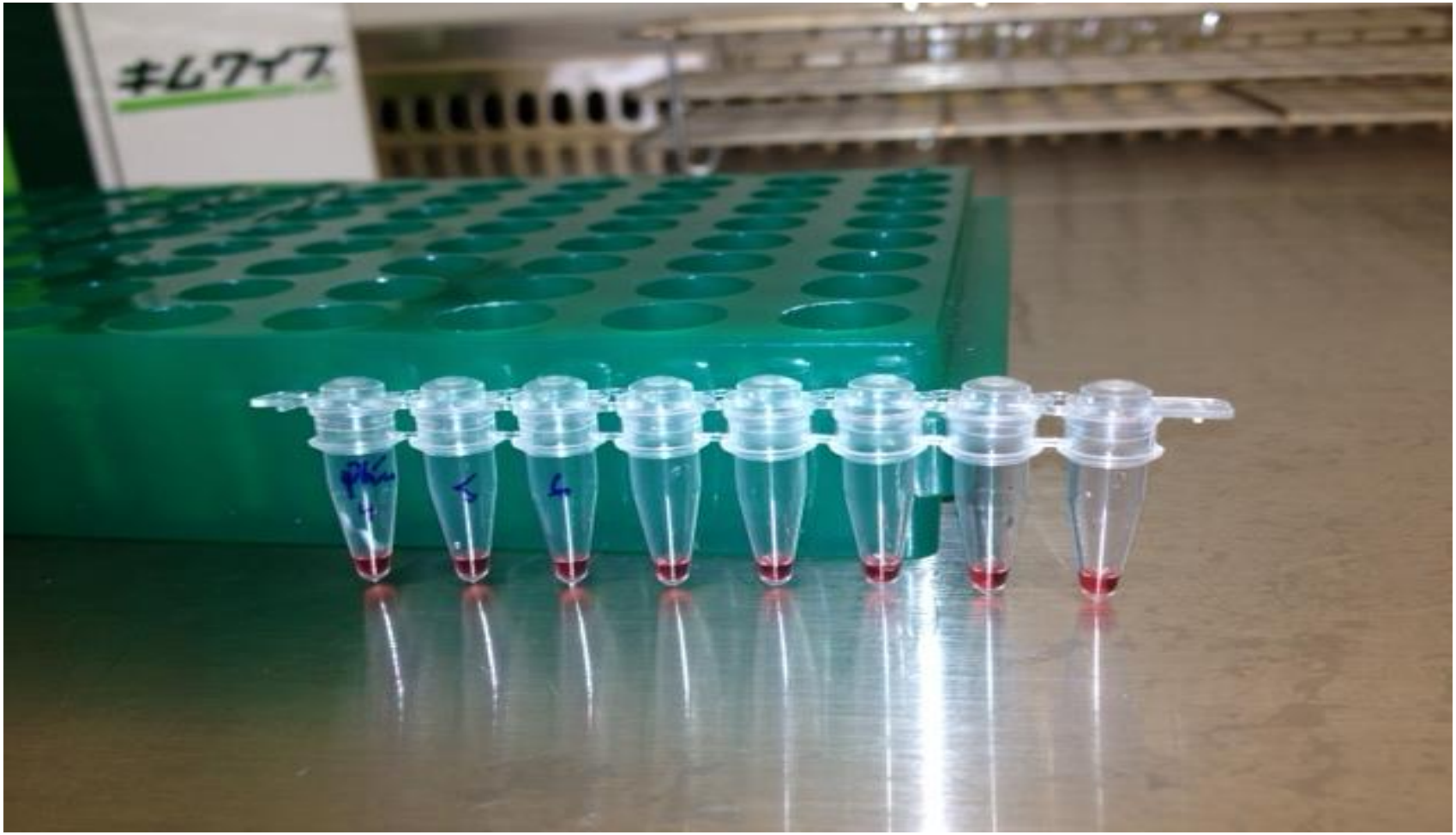
  85°C, 5 s

  Keep 4°C

Screening of ESBL gene expression

- **PCR reaction mixture**

	1 react.	10 react.
Distilled water	1.6 µl	16 µl
2x Multiplex PCR Master Mix	5 µl	50 µl
Q-solution	1 µl	10 µl
Coral Load Dye	1 µl	10 µl
Primer mix ESBL multi V6	0.4 µl	4 µl


キムワイプ

Thermal cycler

- 95°C/5 min
- ***Repeat 30 cycles***
- 95°C/30 s
- 60°C/90 s
- 72°C/90 s

- 68°C/10 min
- Cool down 4-16°C

Singleplex PCR/ *E. coli* 16S rRNA gene

- **PCR reaction mixture**

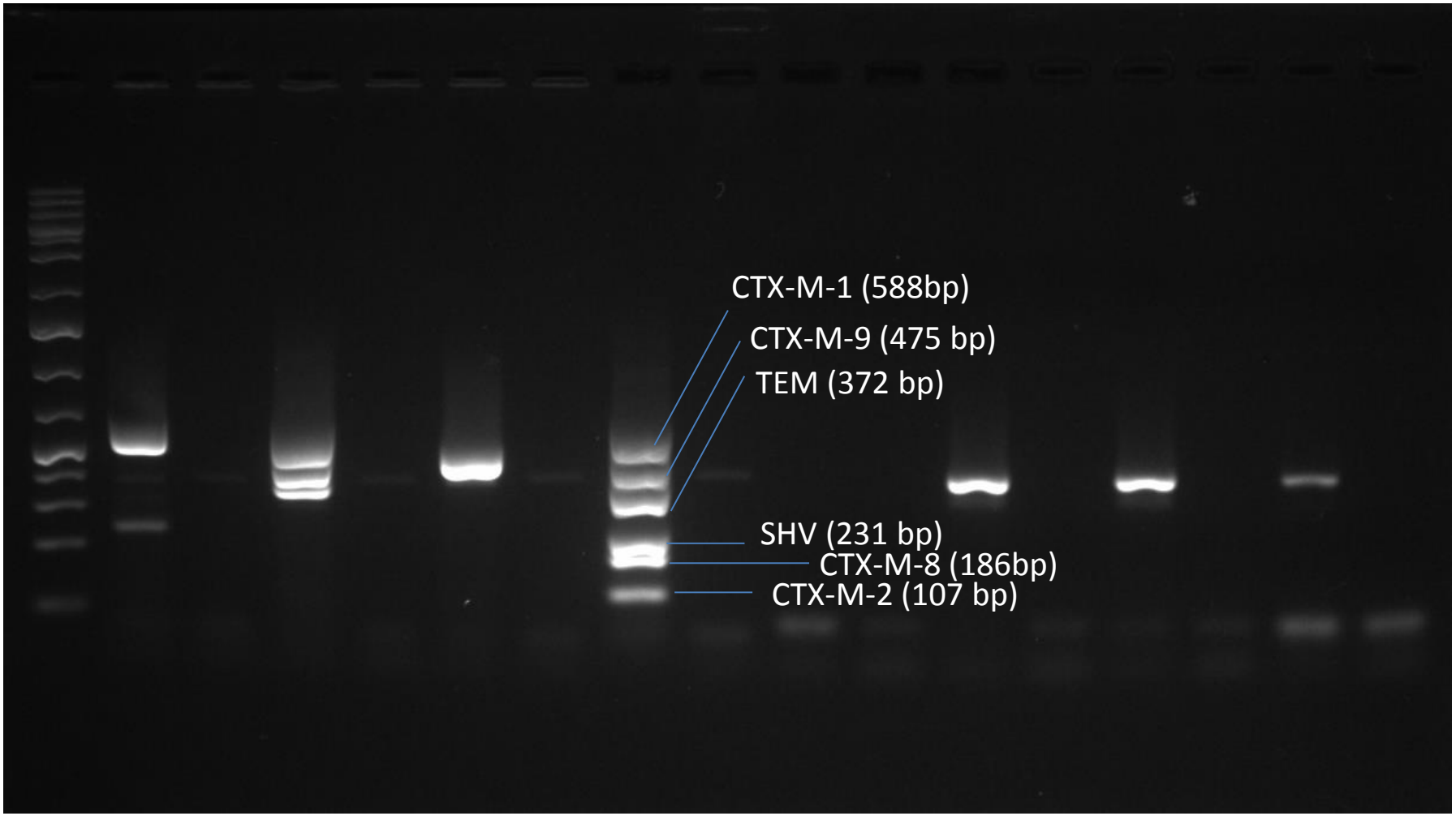
	1 **react.**	**10 react.**
Distilled water	6.75 µl	67.5 µl
10x Ex *Taq* Buffer	1 µl	10 µl
dNTP Mixture	0.8 µl	8 µl
Primer mix (10 µM)	0.4 µl	4 µl
Takara Ex *Taq*	0.05 µl	0.5 µl

Thermal cycler

- 95°C/5 min
- ***Repeat 30 cycles***
- 95°C/10 s
- 60°C/30 s
- 72°C/60 s

- 72°C/7 min
- Cool down 4-16°C

PHONG (10/10)



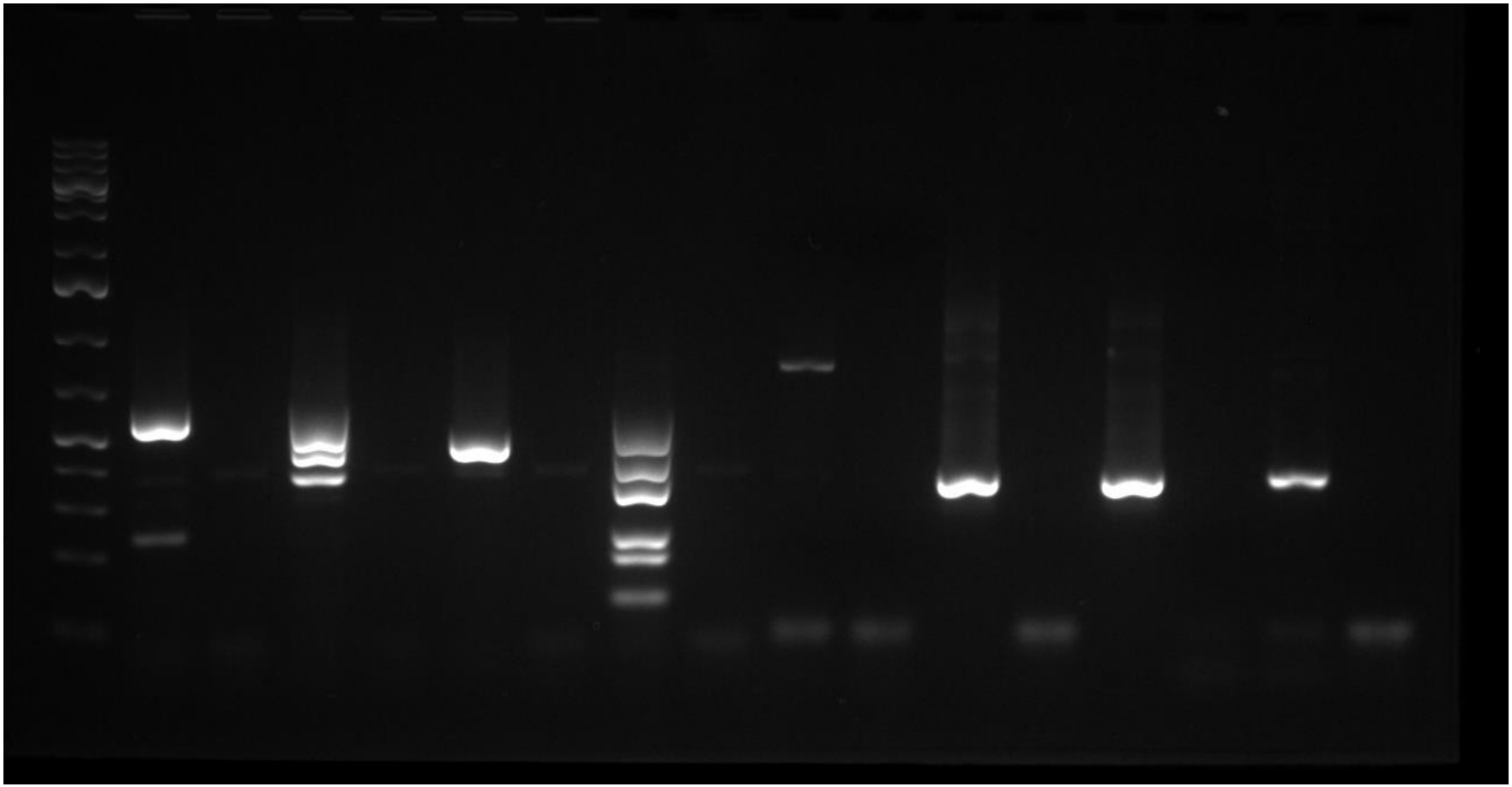
MK 1 1-1 2 2-2 3 3-3 C+ C- 1 1-2 2 2-2 3 3-3 C+ C-

DNA

5µl/sample well

3µl/MK

DUYEN (10/10)



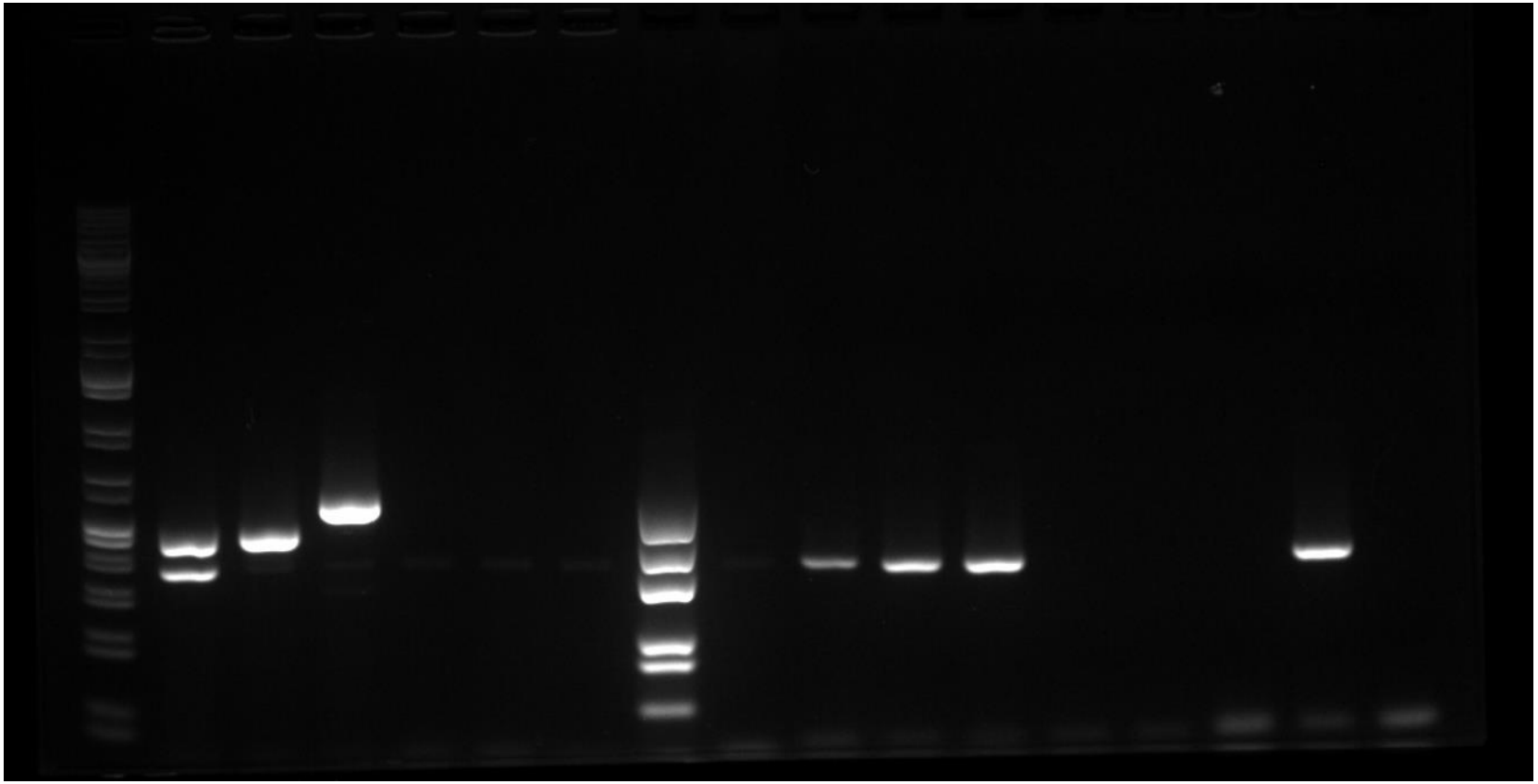
MK 1 1-1 2 2-1 3 3-1 C+ C- 1 1-2 2 2-2 3 3-2 C+ C-

DNA

5µl/sample well

3µl/MK

PHUC (10/10)



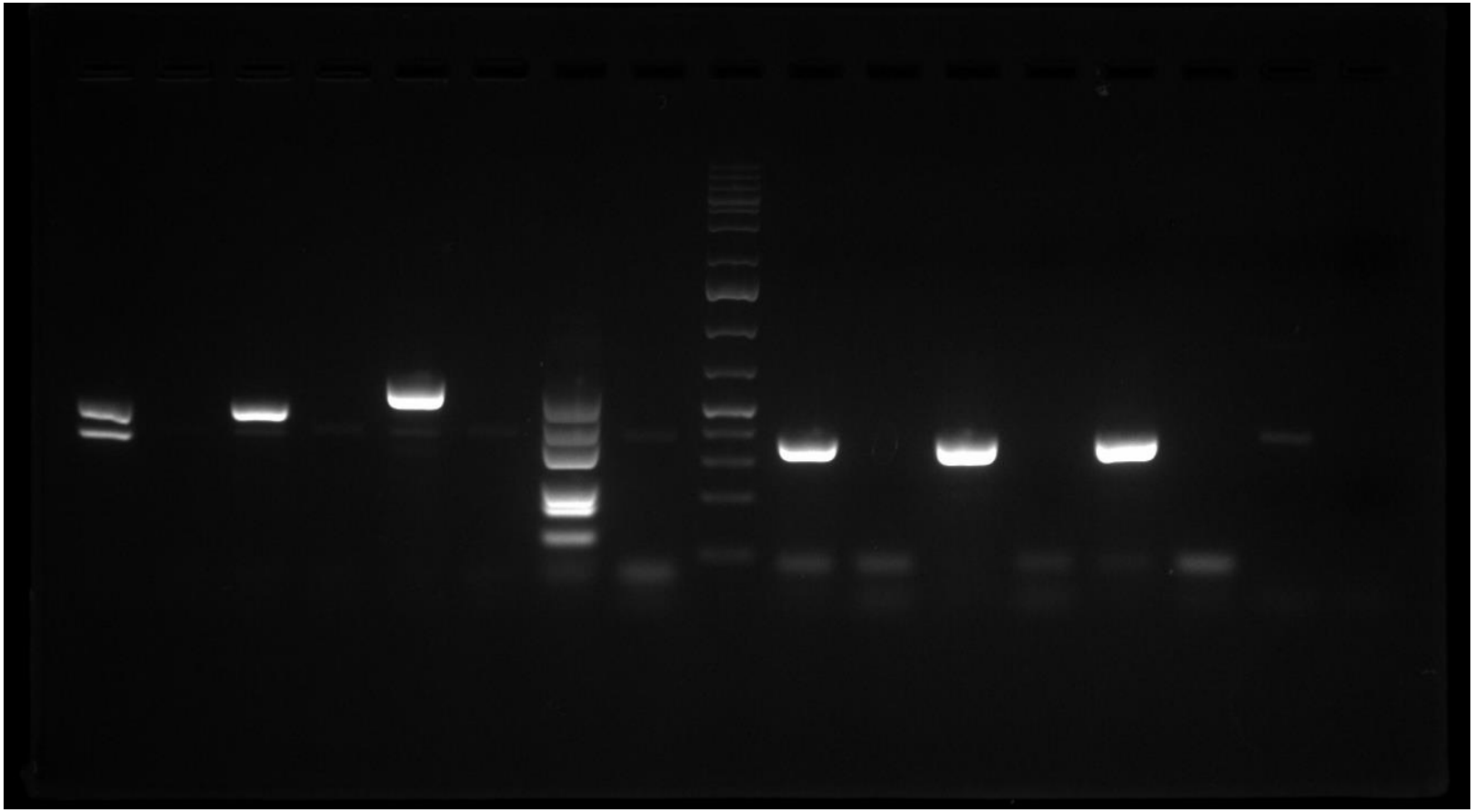
MK 4 5 6 4-1 5-1 6-1 C+ C- 4 5 6 4-2 5-2 6-2 C+ C-

DNA

5µl/sample well

3µl/MK

NGAN (10/10)



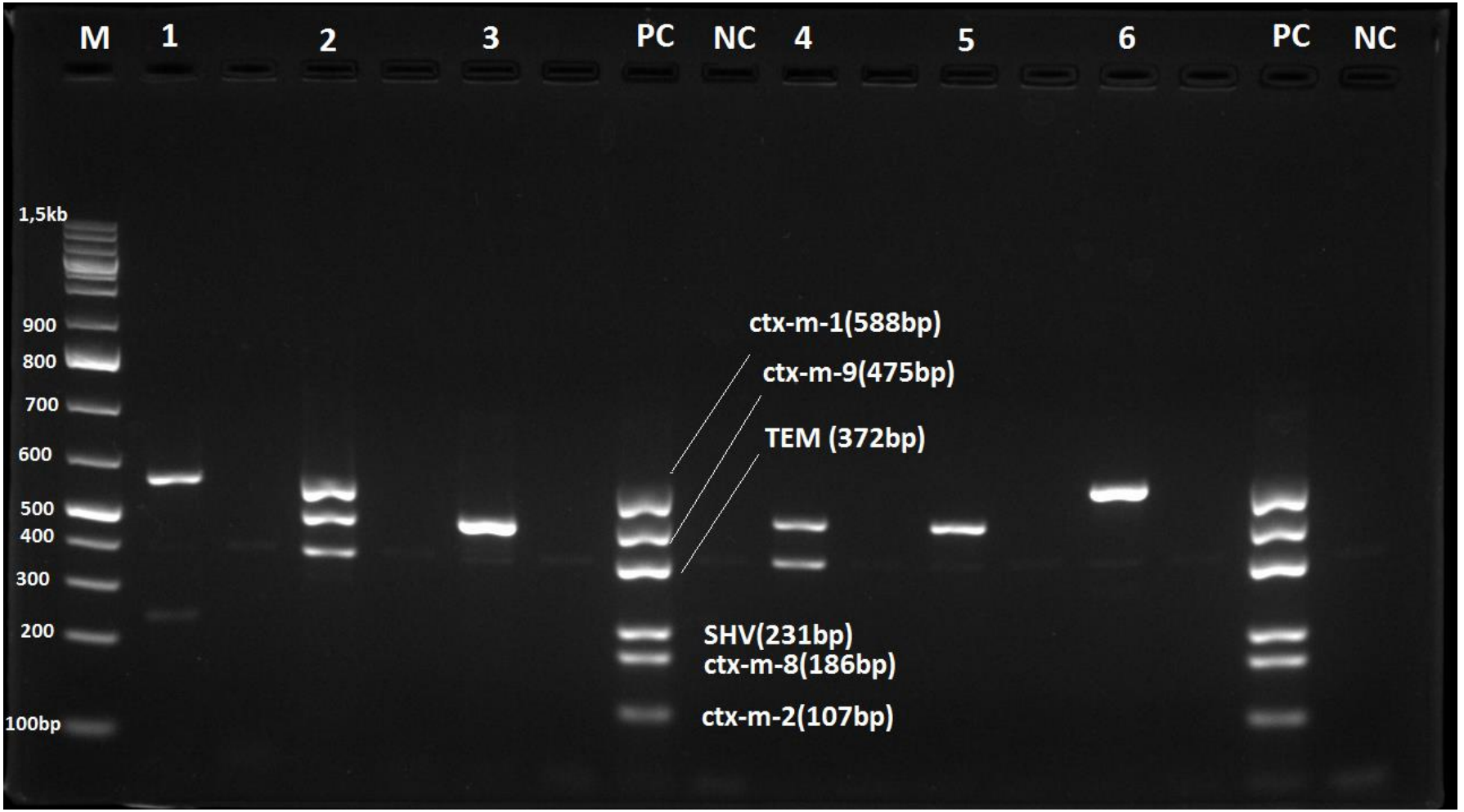
4 4-1 5 5-1 6 6-1 C+ C- MK 4 4-2 5 5-2 6 6-2 C+ C-

DNA

5µl/sample well

3µl/MK

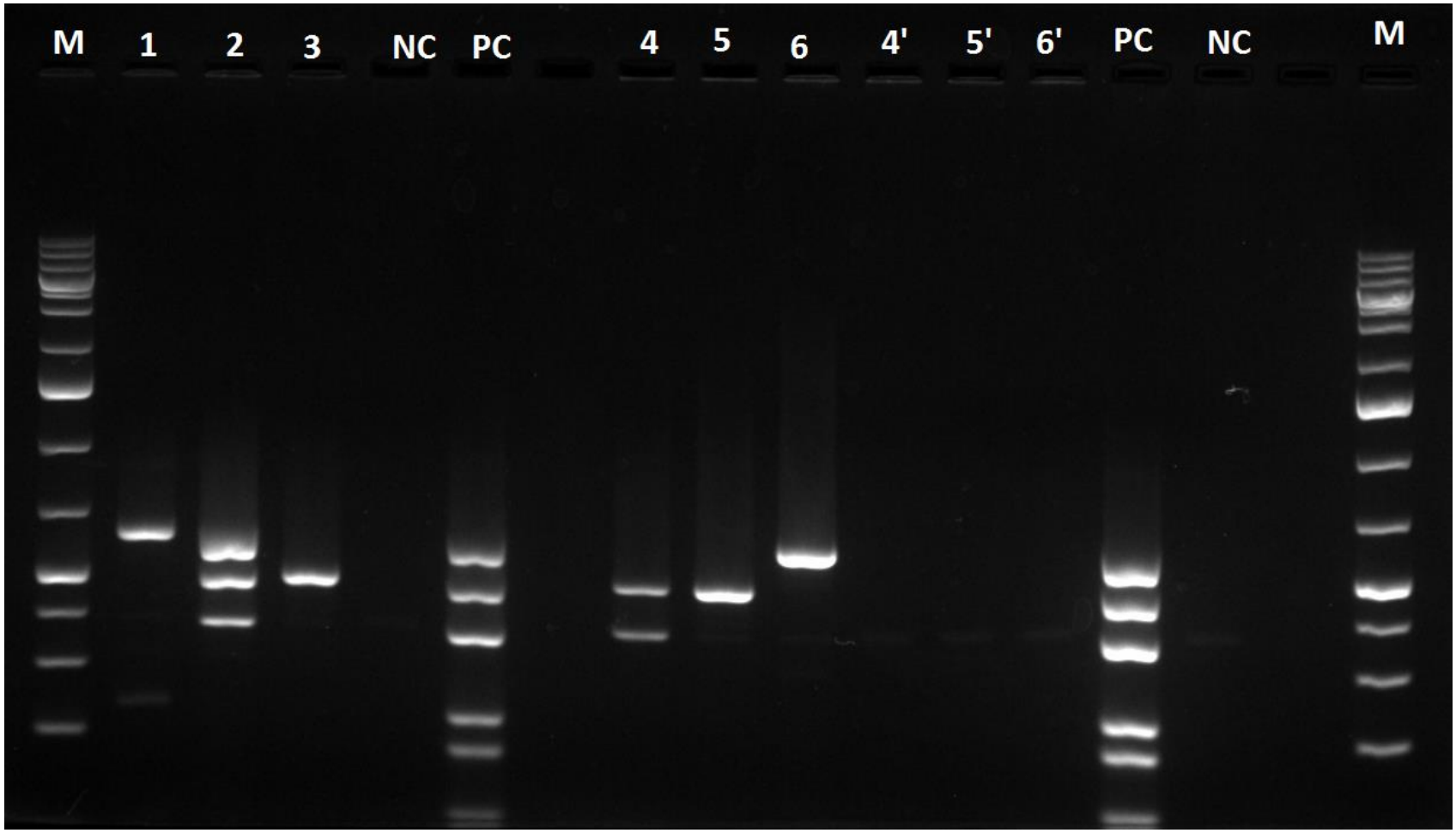
Due to the result of analysis on electrophoresis band on 10/10 are not clear . So date 11/10 PCR products will be diluted and analysis again. The result show that:



Two times dilution PCR products

Phong

Phuc



Three times dilution PCR product

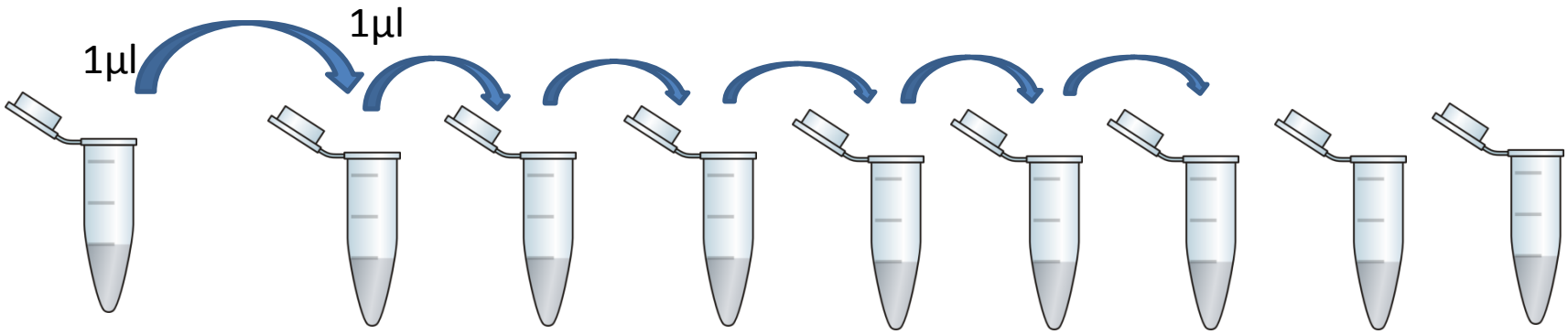
Two times dilution PCR product

Summary of results

No.	Strains	Species	*E. coli* 16S rRNA (cDNA)	ESBL genotyping by multiplex PCR		
				Original information	gDNA (Ueda G)	cDNA
6	08211	*E. coli*	+	CTX-M-1	CTX-M-1	CTX-M-1
5	01122	*E. coli*	+	CTX-M-4	CTX-M-4	CTX-M-4
4	**15322**	***E. coli***	**+**	**All negative**	**CTX-M-4, TEM**	**CTX-M-4, TEM**
3	**06221**	***E. coli***	**+**	**CTX-M-1, 2, 3, 4, SHV, TEM**	**CTX-M-4**	**CTX-M-4**
2	**11111**	***E. coli***	**+**	**CTX-M-1, 4, TEM**	**CTX-M- 4, TEM**	**CTX-M-1, 4, TEM**
1	18111	*K. pneumoniae*	-	CTX-M-1, SHV	CTX-M-1, SHV	CTX-M-1, SHV

Dilution cDNA

- From cDNA



cDNA

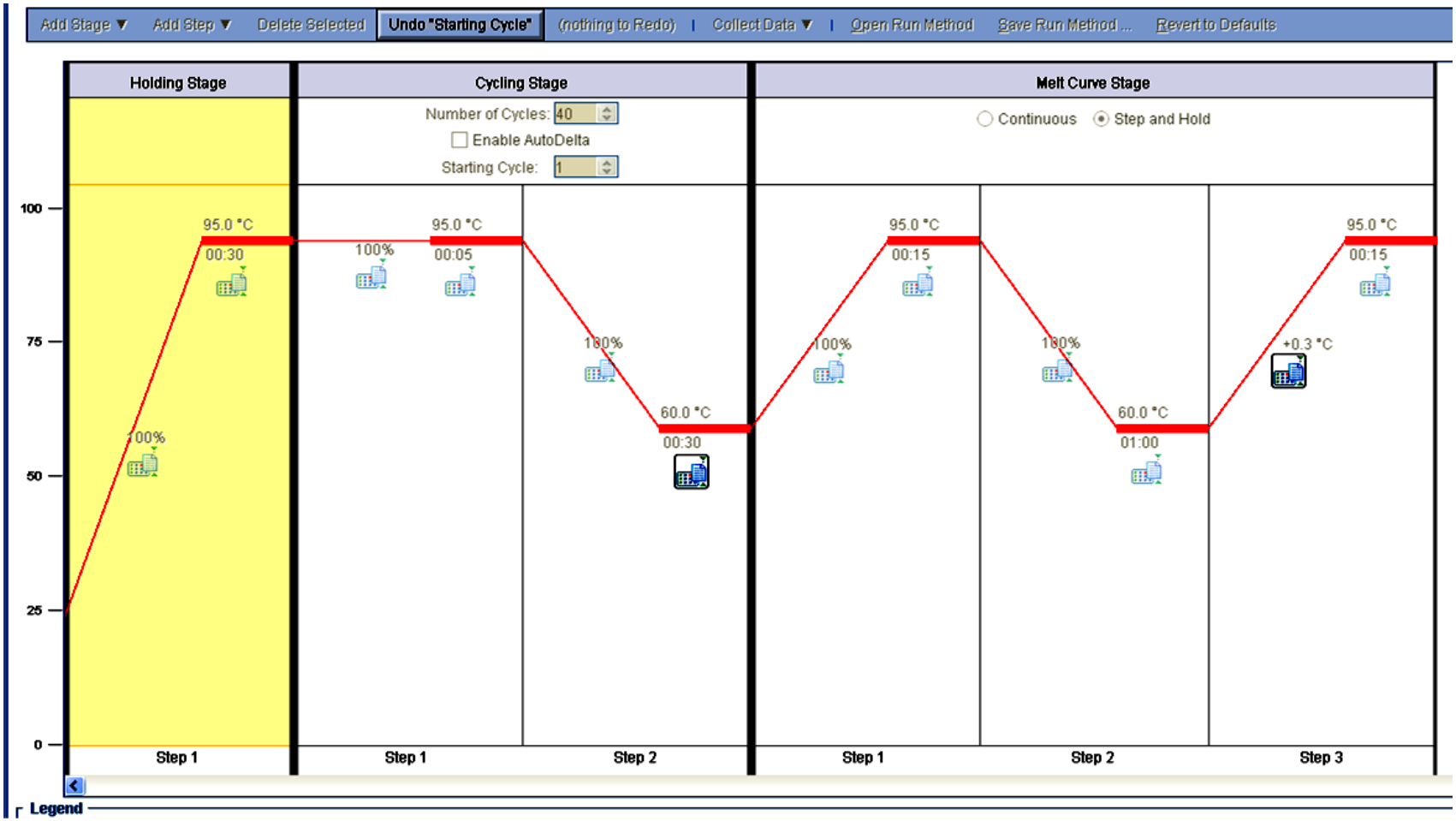
cDNA	1 µl	1 µl	1 µl	1 µl	1 µl	1 µl	10 µl	10 µl
DW	9 µl	9 µl	9 µl	9 µl	9 µl	9 µl		
	10	10^2	10^3	10^4	10^5	10^6	C+	C-

Real time PCR mixture reaction

	1 **react.**	**10 react.**
SYBR *Premix Ex Taq*	10 µl	100 µl
PCR F Primer (10 µM)	0.8 µl	8 µl
PCR R Primer (10 µM)	0.8 µl	8 µl
ROX Ref. Dye or DyeII (50X)	0.4 µl	4 µl
DW	6 µl	60 µl

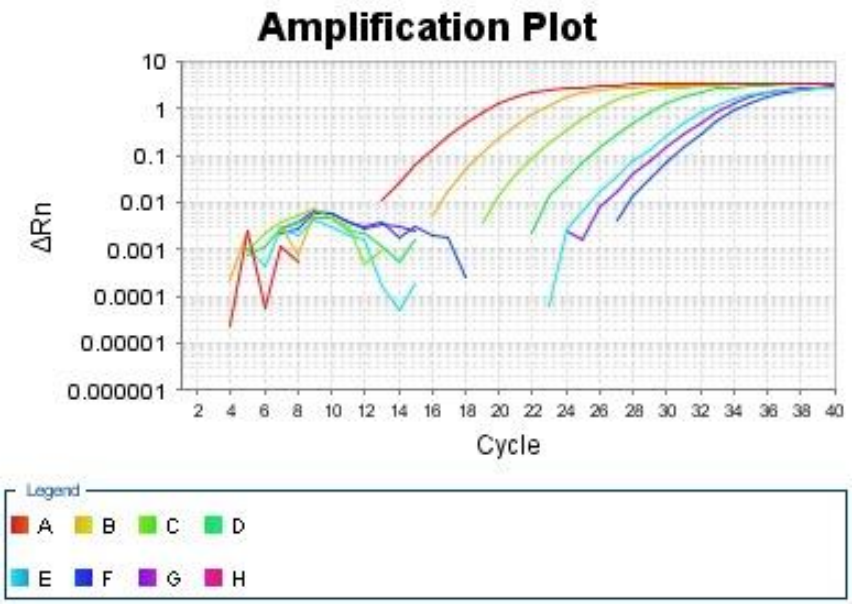
- Amount 1 react. : 20 µl
- 5 µl diluted samples

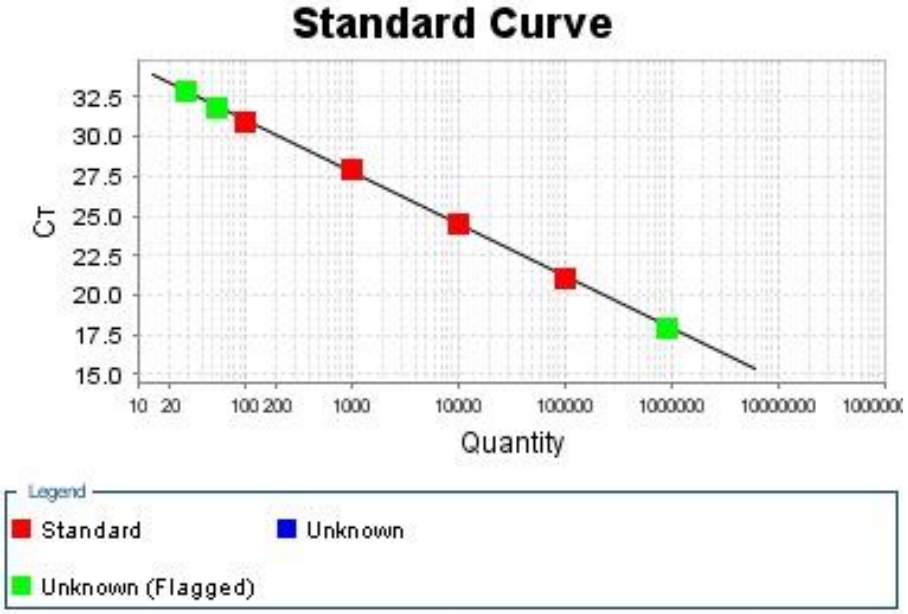
Real Time PCR Protocol



Result the standard cure of cDNA dilution

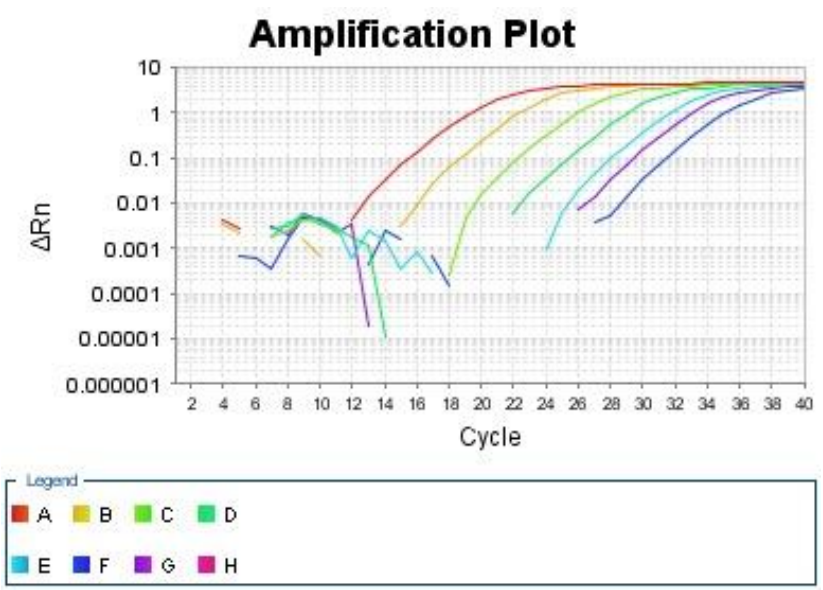
- Phuc: cDNA of sample 4 (15322 *E. coli* ESBL)

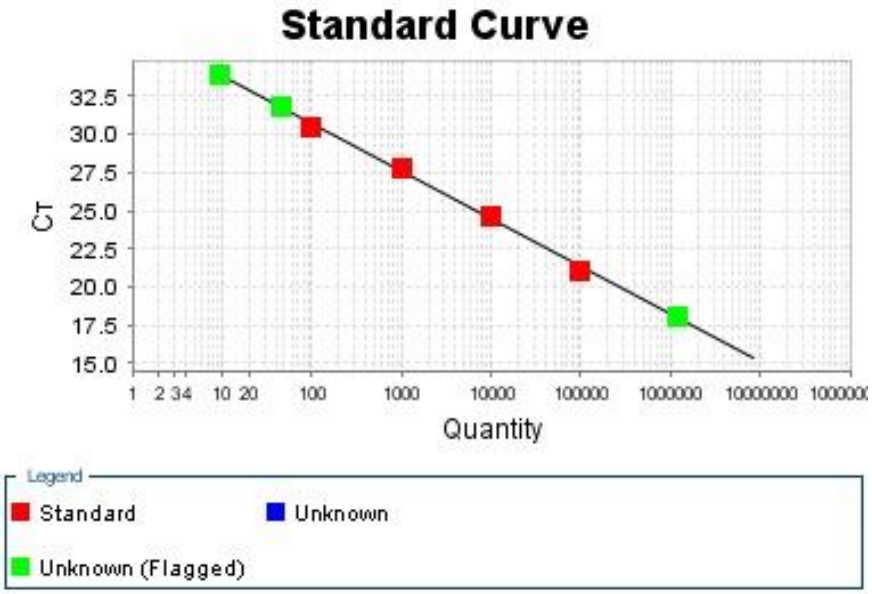




R^2=0.999

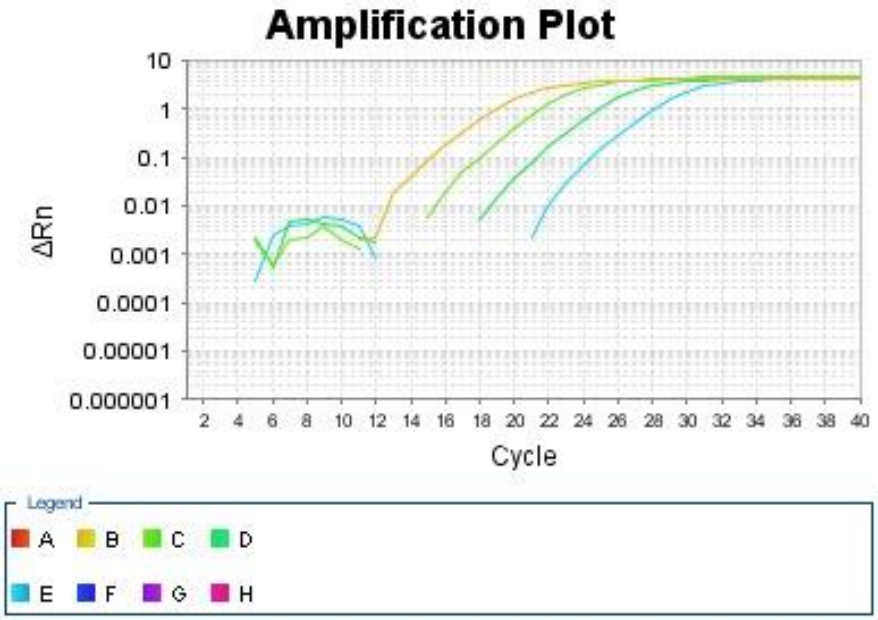
Phong: cDNA of sample 2 (11111 *E. coli* CTX-M1; CTX-M4; TEM)

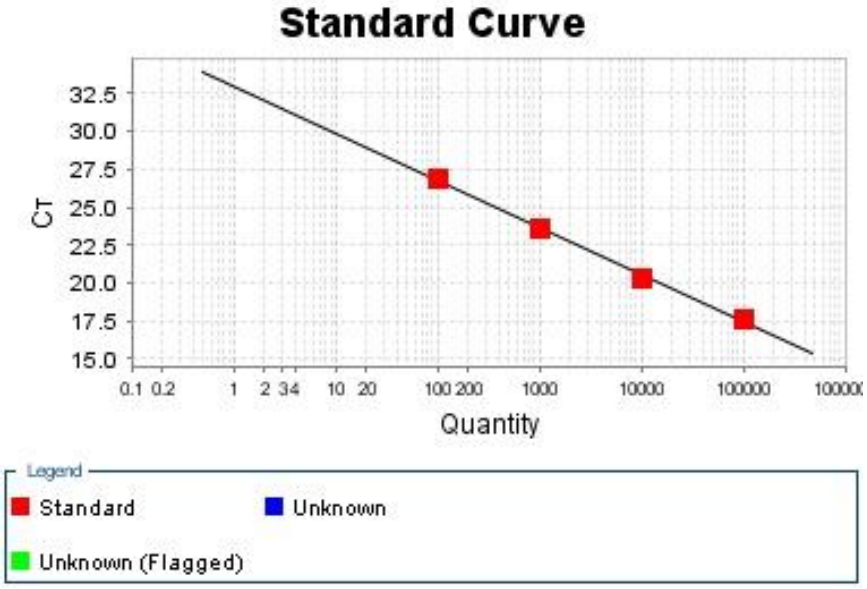




R^2=0.995

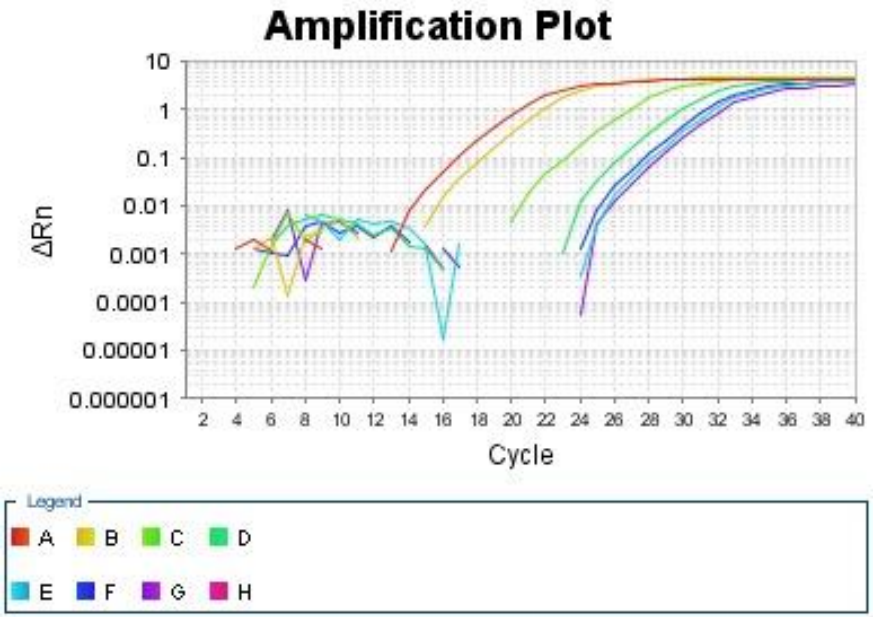
Duyen: cDNA of sample 3 (06221 *E. coli* CTX-M1; CTX-M2; CTX-M3; CTX-M4; SHV; TEM)

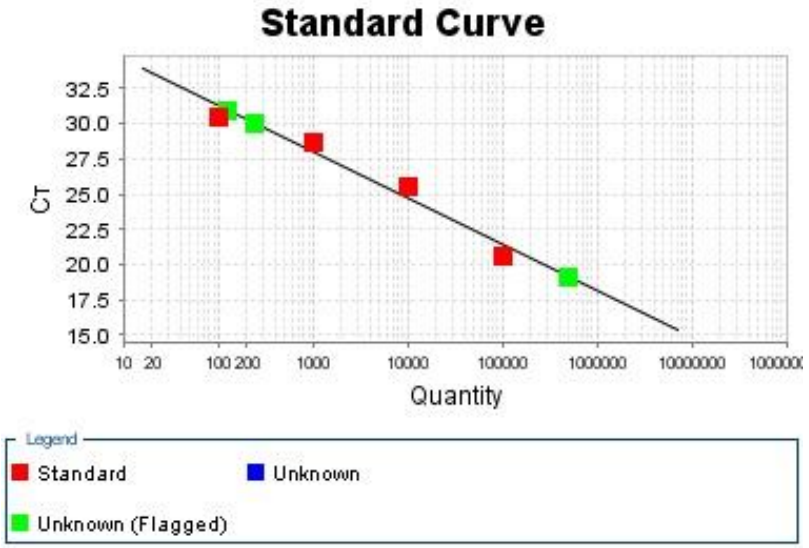




R^2=0.998

Ngan: cDNA of sample 5: 01122: *E. coli* CTX-M4 group





R^2=0.995

LAMP TECHNIQUE

- **Prepare master mix**

- React. Mix Sal (RM Sal) 20 µl
- *Bst* DNA polymerase 1 µl
- Take 20 µl + 5 µl DNA template
- 65^{o}C/60 min

Results of LAMP