PROTOCOL FOR DETECTION OF H7 AVIAN INFLUENZA USING REALTIME RT-PCR
(9 February 2007)

Introduction

HA2 H7 RealTime PCR:

This amplifies within the conserved HA2 region of the H7 haemagglutinin (HA) gene. The primers and probe were designed at VLA (UK) for detection of contemporary Eurasian H7 AIVs, and validation studies with clinical specimens have demonstrated a greater sensitivity than AI isolation in embryonated chickens' eggs.

Primer / probe sequences for HA2 H7 RealTime PCR:

LH6H7:
5'-GGC CAG TAT TAG AAA CAA CAC CTA TGA-3'

RH4H7
5'-GCC CCG AAG CTA AAC CAA AGT AT-3'

H7pro11:

5'-FAM-CCG CTG CTT AGT TTG ACT GGG TCA ATC T-BHQ-3'

This probe is 5' labelled with FAM and 3' labelled with Black Hole Quencher (BHQ).

Cycling conditions

Cycling times & temperatures for the HA2 H7 RealTime PCR are precisely as for the H5 Eurasian RealTime PCR on the Stratagene RealTime PCR platforms, *ie*:

30 mins at 50C, 15 mins at 95C;
Then x40 cycles of:
10 secs at 95C

30 secs at 54C
10 secs at 72C

Fluorescence data is gathered at the end of each 54C annealing step.

As mentioned previously for other avian influenza RealTime PCRs, other instrumentation platforms may require modification of *eg* the cycling conditions to attain optimal H7 sensitivity and specificity.

HA2 H7 RealTime PCR Master Mix:

HA2 H7 RealTime PCR		
Ingredients – all except primers & probe from Qiagen OneStep RT-PCR kit (cat # 210212) & other stated suppliers	Volumes for 10 reaction recipee	Final concentration / strength
DEPC- H_2O (Ambion or similar):	139µl	-
(x5) Qiagen 1-step RT PCR buffer:	50µl	(x1)
ROX ref dye, pre-diluted to 1:500 in DEPC-H_2O from neat stock (Stratagene or similar):	3.75µl	-
Qiagen dNTP mix:	10µl	-
Primer: H7LH6 (50µM):	2µl	0.4µM
Primer: H7RH4 (50µM):	2µl	0.4µM
Probe: H7 Pro11 (30µM):	1.25µl	0.15µM
25mM $MgCl_2$ (Promega or similar):	12.5µl	3.75mM
RNAsin (40U/µl, Promega or similar):	1µl	-
Qiagen 1-step RT PCR enzyme mix:	10µl	-
Total:	**231.5µl**	-

Divide Master Mix into **23µl aliquots** and add **2µl extracted RNA** to each. Cycle as described above for the Stratagene instruments.

MJS
VLA, 9/2/07

Matrix gene universal RealTime Protocol

Primers & hydrolysis ("TaqMan") probe

Primers & probe have been designed originally by Spackman *et al* (J Clin Microbiol (2002) **40;** 3256-3260) as follows:

Forward M gene primer: AGA TGA GTC TTC TAA CCG AGG TCG

Reverse M gene primer: TGC AAA AAC ATC TTC AAG TCT CTG

M gene probe: FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA

Cycling conditions

These are currently in use on Stratagene Mx3000 & Mx4000 instruments at VLA:

30 mins at 50°C, 15 mins at 95°C;
Then x40 cycles of:
10 secs at 95C
20 secs at 60C

NB: Fluorescence data is gathered at the end of each 60C annealing step.
The above conditions work successfully using Stratagene Mx3000/4000 RealTime platforms at VLA, but cycling times, ramp speeds and temperatures may have to be modified if using a different make of RealTime instrument.

Matrix gene RealTime PCR Master Mix

This is based on the Methods section of Spackman *et al* (2002), which utilises the OneStep RT-PCR Kit(Cat # 210212, Qiagen). Reagents not provided in the kit can be obtained from other suppliers as indicated in below table which describes an M gene RealTime Master Mix for 10 x 25ul reactions:

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Ingredients - all except primers & probe are from Qiagen OneStep RT-PCR Kit (cat # 210212) & other stated suppliers:	Volumes (ul) for 10 reaction recipe:
DEPC-H2O (Ambion):	137.5
(x5) Qiagen 1-step RT PCR buffer:	50
ROX ref dye (*ie* 1:500 dilution of neat Stratagene stock in DEPC-H2O):	3.75
Qiagen dNTP mix:	10
Forward M gene primer (50uM):	2
Reverse M gene primer (50uM):	2
M gene probe (30uM): (Remember to reconstitute freeze-dried probe in (x1) TE buffer*)	2.5
25mM MgCl2 (Promega):	12.5
RNAsin (40U/ul, Promega):	1
Qiagen 1-step RT PCR enzyme mix:	10
Total	231.25

Mix the Master Mix tube thoroughly by inversion & flicking, spin approx 30" to remove bubbles.
Then aliquot 23ul per well of Mx3000/4000 plate, then add 2ul extracted RNA as target.

*TE buffer (x1): 10mM Tris-HCl (pH7.5) 1mM EDTA

1. Preparation of PCR master mix and loading of real time plate to be carried out in the PCR clean room. Once loaded, place caps loosely on the wells of the plate and bring out of the clean room.

2. Remember to include an AI positive control RNA for each M gene RealTime experiment. Positive control RNA to be tested in duplicate in each experiment.

3. Depending on the number of samples, load samples manually or by using a liquid-handling robot.

4. Once RNA is loaded onto the plate, ensure caps are pushed firmly onto the wells.

5. Keep plate on ice if there is going to be a delay in testing on the Mx3000/4000 RealTime instrument.

SE, VLA

N1 AIV GENE DETECTION BY REALTIME PCR

Current version at VLA: 6 February 2007

Primers and hydrolysis ("TaqMan") MGB probe

These are presently as designed by Payungporn *et al* (J Virol Methods (2006) **131**;143-147):

N1F2 (forward primer): 5'-GTT TGA GTC TGT TGC TTG GTC-3'

N1R1: (reverse primer): 5'-TGA TAG TGT CTG TTA TTA TGC C-3'

N1 probe: 5' VIC-TTG TAT TTC AAT ACA GCC AC-MGB 3' (Please note this is an MGB probe)

Payungporn *et al* originally utilised a NED-labeled MGB probe, but experiments at VLA have demonstrated successful N1 detection with the VIC-labeled MGB probe. FAM-labeled N1 probes should also be successful. **NB**: MGB probes require ordering from ABI. ABI supply N1 probe as a 100µM solution in (x1) TE buffer, so *eg* take 30µl of this and add 70µl DEPC-H_2O to make a 30µM strength N1 probe. Divide into smaller aliquots (*eg* 10-20 µl) and store frozen.

Cycling conditions

These are currently in use on Stratagene Mx3000 & Mx4000 instruments at VLA. Other laboratories using different instrumentation platforms should first critically and carefully examine these cycling conditions as they may ***not*** perform optimally on non-Stratagene RealTime instruments. Hence cycling temperatures, times and ramp speeds may all need to be modified by a non-Stratagene user:

30 mins at 50C, 15 mins at 95C;
Then x40 cycles of:
15 secs at 95C
30 secs at 55C
40 secs at 72C

NB: Fluorescence data is gathered at the end of each 55C annealing step. *Select HEX channel for N1 detection and ROX channel for the reference dye.*

N1 RealTime PCR Master Mix

This protocol has been adapted to the OneStep RT-PCR Kit (Cat # 210212, Qiagen). Reagents not provided in the kit can be obtained from other suppliers as indicated in below table which describes an N1 RealTime Master Mix for 10 x 25ul reactions:

Ingredients - all except primers & probe are from Qiagen OneStep RT-PCR Kit (cat # 210212) & other stated suppliers:	Volumes (ul) for 10 reaction recipe:
DEPC-H2O (Ambion):	139
(x5) Qiagen 1-step RT PCR buffer:	50
ROX ref dye (neat stock from Stratagene pre-diluted to 1:500 in DEPC-H2O):	3.75
Qiagen dNTP mix:	10
N1F2 (50uM):	2
N1R1 (50uM):	2
N1 probe (30uM), ie VIC-labelled MGB probe: (Remember to dilute 100uM stock from ABI as described above)	1.25
25mM MgCl2 (Promega):	12.5
RNAsin (40U/ul, Promega):	1
Qiagen 1-step RT PCR enzyme mix:	10
Total	231.25

Mix the Master Mix tube thoroughly by inversion & flicking, spin approx 30" to remove bubbles.
Then aliquot 23ul per well using P100, then add 2ul extracted RNA as target.

Current status of the N1 RealTime PCR at VLA

While formal validation of this N1 RealTime PCR has not been completed at VLA at the time of writing, this assay has demonstrated a high degree of sensitivity and specificity for the rapid detection of N1. A greater range of N1 subtypes has been tested and successfully detected than described in the original publication of Payungporn *et al* (above). Representatives of the eight non-N1 subtypes have shown that this assay should not generate false-positives. In addition, avian-like swine N1 influenza A viruses are detected by this N1 RealTime PCR.

MJS
VLA 6/2/07

Allegato 2
Risultati delle prove effettuate sulle piattaforme mobili Rapid System, presso Nucleo NBCR

RT GeneM VLA

EID50/100ml	10e4,375	10e3,375	10e2,375	10e1,375	10e0,375	10e-0,625
I serie RT1	24,81	26,72	30,99	34,15		
	24,17	26,95	31,31	34,99		
	24,11	26,61	31,05	33,09		
MEDIA	24,36	26,76	31,12	34,08		
DEV. STAND.	0,39	0,17	0,17	0,95		
COEFF.di VAR.%	1,59	0,65	0,55	2,79		

I serie RT2	22,94	24,56	29,62	31,78		
	23,44	25,93	29,91	26,89		
	21,44	24,78	29,61	31,82		
MEDIA	22,61	25,09	29,71	30,16		
DEV. STAND.	1,04	0,74	0,17	2,83		
COEFF.di VAR.%	4,60	2,93	0,57	9,40		

II serie RT2	23,35	27,76	29,21	32,66	35,82	
	23,98	27,24	28,88	32,15		
	22,76	26,64	28,40	31,91	35,68	
MEDIA	23,36	27,21	28,83	32,24	35,75	
DEV. STAND.	0,61	0,56	0,41	0,38	0,10	
COEFF.di VAR.%	2,61	2,06	1,41	1,19	0,28	

II serie RT2	27,33	31,09	33,05			
	27,52	31,44	33,84			
	27,56	31,60	32,67			
MEDIA	27,47	31,38	33,19			
DEV. STAND.	0,12	0,26	0,60			
COEFF.di VAR.%	0,45	0,83	1,80			

Data emissione: 20-07-2009	Revisione: 1 N° pag complessive:	Autori: Cristian De Battisti, Dipartimento Ricerca e Sviluppo, SC6, IZS Venezie, Legnaro (PD), cdebattisti@izsvenezie.it Salvatore Minghetti Ministero dell'Interno Dipartimanto dei Vigili del Fuoco del Soccorso Pubblico e della Difesa Civile Comando Provinciale VV.F. di Venezia, salvatore.minghetti@vigilfuoco.it

RT H5 VLA

EID50/100ml	10e4,83	10e3,83	10e2,83	10e1,83	10e0,83	10e-0,17
I serie RT1	20,09	23,42	28,79	32,40	36,32	
	19,92	20,91	27,62	32,39	34,91	
	19,91	23,76	27,41	32,22	36,54	
MEDIA	19,97	22,70	27,94	32,34	35,92	
DEV. STAND.	0,10	1,56	0,74	0,10	0,88	
COEFF.di VAR.%	0,51	6,86	2,66	0,31	2,46	

I serie RT2	19,59	24,25	28,68			
	21,17	24,51	28,76			
	21,28	24,41	28,73			
MEDIA	20,68	24,39	28,72			
DEV. STAND.	0,95	0,13	0,04			
COEFF.di VAR.%	4,57	0,54	0,14			

II serie RT1	18,83	24,70	25,71	34,84		
	18,77	24,52	25,42	35,82		
	19,39	24,31	27,11			
MEDIA	19,00	24,51	26,08	35,33		
DEV. STAND.	0,34	0,20	0,90	0,69		
COEFF.di VAR.%	1,80	0,80	3,47	1,96		

II serie RT2	17,82	23,85	24,37	31,16	33,93	
	18,10	23,48	24,93	30,93	34,81	
	18,48	23,82	25,38	31,96	33,90	
MEDIA	18,13	23,72	24,89	31,35	34,21	
DEV. STAND.	0,33	0,21	0,51	0,54	0,52	
COEFF.di VAR.%	1,83	0,87	2,03	1,72	1,51	

RT H7 VLA

EID50/100ml	10e4,375	10e3,375	10e2,375	10e1,375	10e0,375	10e-0,625
I serie RT1	27,77	30,76	34,74	34,44		
	28,22	30,48	34,01	36,36		
	27,86		34,52	37,85		
MEDIA	27,95	30,62	34,42	36,22		
DEV. STAND.	0,24	0,20	0,37	1,71		
COEFF.di VAR.%	0,85	0,65	1,09	4,72		

I serie RT2	26,04	29,28	32,83	33,59		
	26,91	29,57	31,95	34,36		
	26,31	28,07	32,98	35,77		
MEDIA	26,42	28,97	32,59	34,57		
DEV. STAND.	0,45	0,80	0,56	1,11		
COEFF.di VAR.%	1,69	2,75	1,71	3,20		

II serie RT1	28,15	31,15	33,80	38,25		
	26,89	31,82	33,66	35,43		
	27,85	31,89	33,88	36,65		
MEDIA	27,63	31,62	33,78	36,78		
DEV. STAND.	0,66	0,41	0,11	1,41		
COEFF.di VAR.%	2,38	1,29	0,33	3,85		

II serie RT1	25,85	30,32	32,56	36,03	35,96	
	25,41	30,50	32,53	35,64		
	26,53	25,87	32,79	34,73		
MEDIA	25,93	28,90	32,63	35,47		
DEV. STAND.	0,56	2,62	0,14	0,67		
COEFF.di VAR.%	2,18	9,08	0,44	1,88		

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RT N1 VLA

EID50/100ml	10e4,83	10e3,83	10e2,83	10e1,83	10e0,83	10e-0,17
I serie RT1	18,90	21,96	28,06	31,10	35,96	
	19,48	22,42	27,85	31,97	33,36	
	18,72	22,47	27,36	31,87	33,90	
MEDIA	19,03	22,28	27,76	31,65	34,41	
DEV. STAND.	0,40	0,28	0,36	0,48	1,37	
COEFF.di VAR.%	2,09	1,26	1,29	1,50	3,99	

I serie RT2	22,40	25,78	29,56	31,92		
	21,66	24,82	30,15	33,77		
	20,33	24,02	29,68	32,47		
MEDIA	21,46	24,87	29,80	32,72		
DEV. STAND.	1,05	0,88	0,31	0,95		
COEFF.di VAR.%	4,89	3,54	1,05	2,90		

I serie RT2	22,19	26,05	29,94	33,43	37,75	
	22,81	25,24	30,71	33,65	37,35	
	22,34	25,93	30,69	33,26		
MEDIA	22,45	25,74	30,45	33,45	37,55	
DEV. STAND.	0,32	0,44	0,44	0,20	0,28	
COEFF.di VAR.%	1,44	1,70	1,44	0,58	0,75	

II serie RT2	19,75	23,71	22,95	31,71	34,24	
	17,37	24,83	25,70	32,23	32,58	
	19,55	24,71	25,44	31,87	33,82	
MEDIA	18,89	24,42	24,70	31,94	33,55	
DEV. STAND.	1,32	0,61	1,52	0,27	0,86	
COEFF.di VAR.%	6,99	2,52	6,15	0,83	2,57	

Allegato 3:
Risultati delle prove effettuate sulla pittaforma Light Cycler, presso IZSVe

RT GeneM VLA

EID50/100ml	10e4,375	10e3,375	10e2,375	10e1,375	10e0,375	10e-0,625
I serie RT3 IZSVe	23,83	27,44	29,38	33,15	35,81	
	23,67	27,68	29,70	33,01		
	23,67	27,55	29,44	34,11	35,88	
MEDIA	23,72	27,56	29,51	33,42	35,85	
DEV. STAND.	0,09	0,12	0,17	0,60	0,05	
COEFF.di VAR.%	0,39	0,44	0,58	1,79	0,14	

RT H5 VLA

EID50/100ml	10e4,83	10e3,83	10e2,83	10e1,83	10e0,83	10e-0,17
I serie RT3 IZSVe	18,56	23,91	24,80	30,81	31,48	31,83
	18,56	23,92	24,76	30,67	31,24	31,06
	18,57	23,86	24,72	30,69	31,10	
MEDIA	18,56	23,90	24,76	30,72	31,27	31,45
DEV. STAND.	0,01	0,03	0,04	0,08	0,19	0,54
COEFF.di VAR.%	0,03	0,13	0,16	0,25	0,61	1,73

RT H7 VLA

EID50/100ml	10e4,375	10e3,375	10e2,375	10e1,375	10e0,375	10e-0,625
I serie RT3 IZSVe	25,81	28,82	31,88	34,44		
	25,93	28,21	31,69	36,36		
	26,19	28,83	32,25	37,85		
MEDIA	25,98	28,62	31,94	36,22		
DEV. STAND.	0,19	0,36	0,28	1,71		
COEFF.di VAR.%	0,75	1,24	0,89	4,72		

RT N1 VLA

EID50/100ml	10e4,83	10e3,83	10e2,83	10e1,83	10e0,83	10e-0,17
I serie RT3 IZSVe	21,43	24,45	29,07	33,32	36,76	
	21,54	24,39	29,15	33,19	35,67	
	21,38	24,34	29,54	33,04	35,27	39,42
MEDIA	21,45	24,39	29,25	33,18	35,90	39,42
DEV. STAND.	0,08	0,06	0,25	0,14	0,77	
COEFF.di VAR.%	0,38	0,23	0,86	0,42	2,15	

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Firefighters, Public Rescue and Civil Defence Department - Italy

First analysis in emergency conditions for ricin toxin detection

Efficency tests on ricin toxin biostrips

Francesco Pilo

Salvatore Minghetti

Provincial Firefighters Headquarter of Venice

Ministry of the Interior

EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Purpose

One of the most important difficulty during a suspect biological emergency is a quick first level detection of some of most critical biological agents.

Work's purposes are to define in case of ricin toxin presence:

- *Real efficency of biostrip results*
- *Detection limits of biostrips in order to sample condition*
- *Create valid procedures for sampling in order to have a reliable detection with biostrip*
- *Define operating procedures for test with biostrips*

EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Test objectives

- *Define utilization area of biostrip*
- *Define limits of biostrip use during on-site analysis*
- *Define a procedure of sample compatible with a complete operators safety. Study these procedures for the correct use in a biological mobile lab.*
- *Define the possibility and incidence of positive/negative false results*

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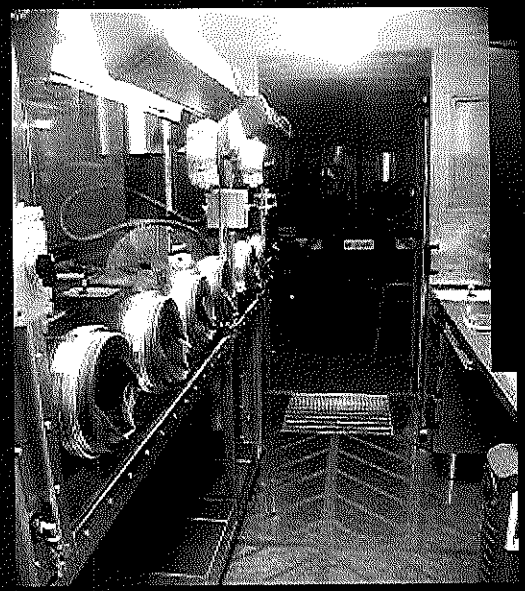
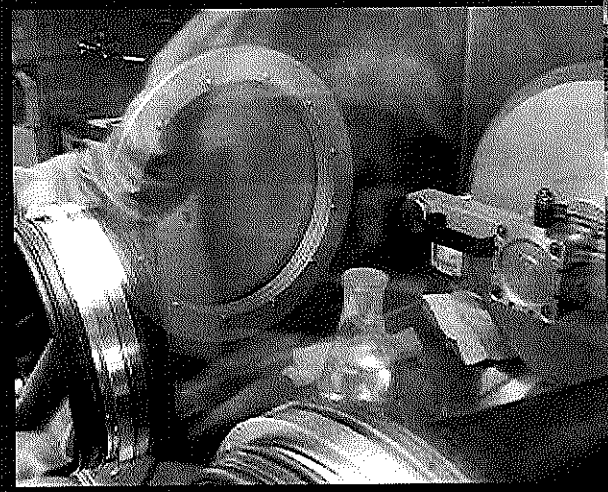
EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Experience Area



mobile biological lab for on-site analysis



Glove boxes system for complete safety of operator during manipulation and sample processing

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EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

On-site analysis:

Integrated procedures

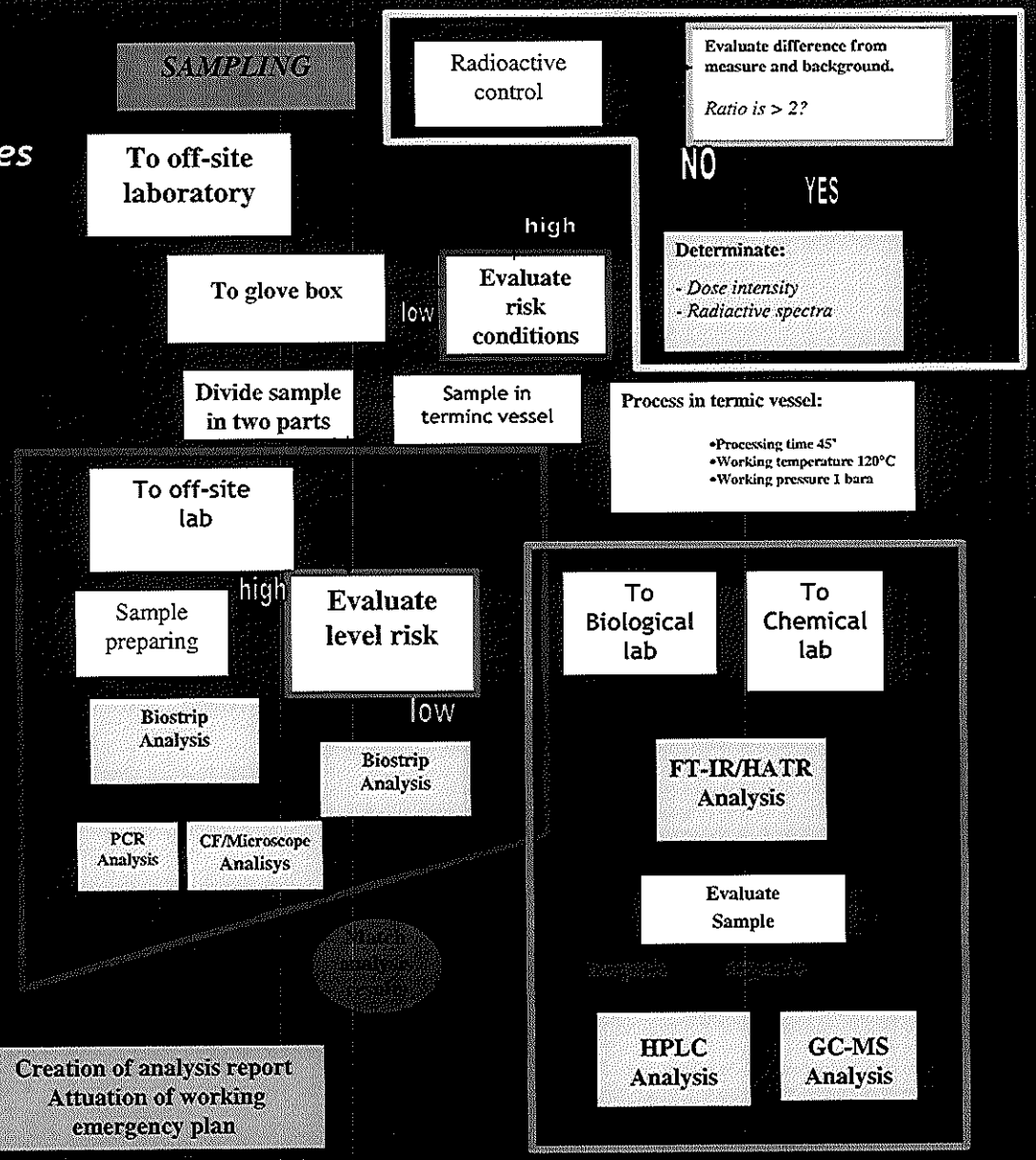
Radiactive analysis

Biological analysis

Chemical analysis

1° detection level

2° detection level



EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Principle of the Bio Detect Test strip

The Bio Detect Test strip is a lateral-flow immunochromatographic device that uses two antibodies in combination to specifically detect the antigen (in this case ricinus communis agglutinin II) in solution. One of the antigen-specific antibodies is labeled with a colloidal gold derivative.

Sample mixes with the colloidal gold-labeled antibody and the move along the strip membrane via capillary action.

If the antigen is present a second antigen-specific antibody captures the colloidal gold-labeled antibody forming a colored line in sample window.

Type of biostrip used during these tests:

Alexeter Technologies LLC Commercial Biological Defense Systems

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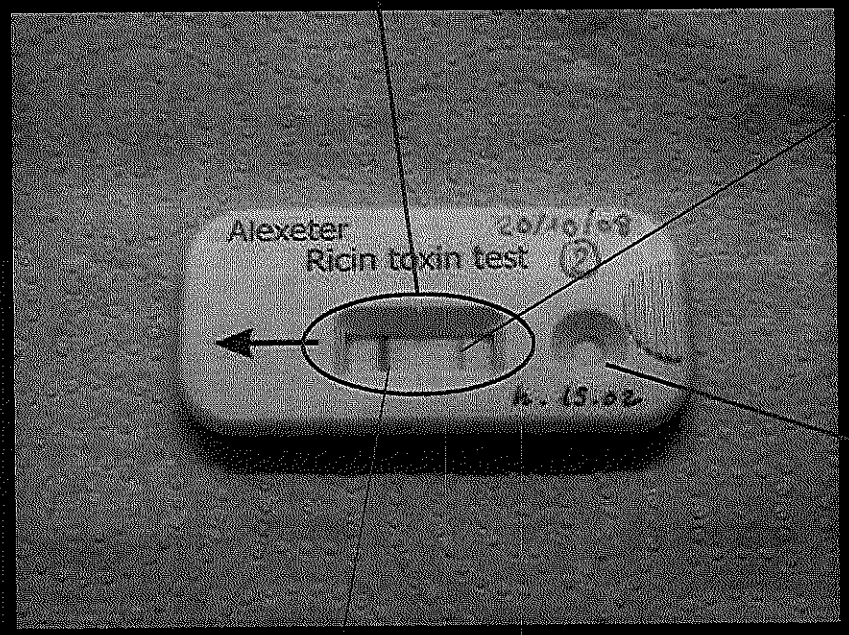
EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Bio Detect Test Strip



sample window

sample line

sample port

control line

EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Sample processing during tests

1° step: Inlet biological laboratory: ricin seeds first treatment

- Calculate seed's weight and seeds measures eliminate external seed' coat and introduce sample inside glove box system

•2° step: Sample preparing

- Preparation of seeds pulp with cutting and homogenization (1 seeds/1 ml H2O)
- Dispersion Homogenized sample into liquid or solid matrix (H2O, foods, drinks)
- External decontamination of the sample and introduction inside a biological static cap

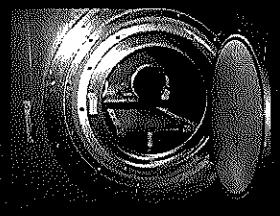
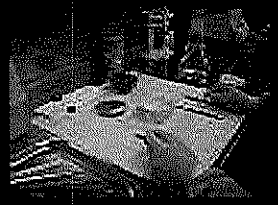
3° step: Immunological Analysis

- Fill the sample vial with buffer solution
- Add sample to vial
- Vortex for ten seconds
- Remove liquid from top layer of vial
- Evaluate PH (outside 4-10 range operate 1:40 diluition)
- Put 0.15 ml in sample port of biostrip
- Evaluate test strip response after 15 minutes

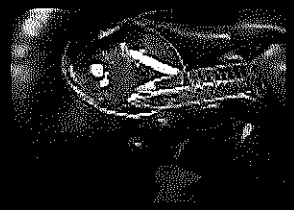
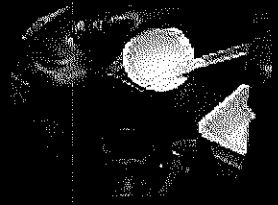
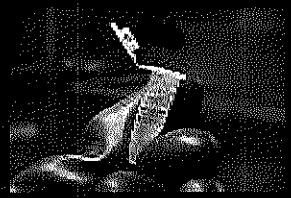
EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Sample metodology treatment

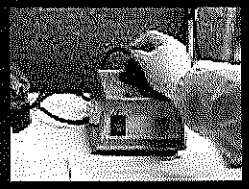
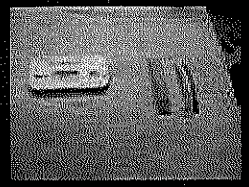
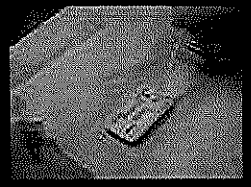
1° step: Inlet biological laboratory: ricin seeds first treatment

2° step: Sample preparation

3° step: Immunological Analysis

EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Complexive program

- Total number of seeds: 273
- Complete registration of measures on 50 seeds representative pool
- Complexive 50 biostreep tests
- Immunological tests on liquid preparations
- Immunological tests on solid preparations
- Determination of incidence of positive/negative false results
- Possibility of cross reaction with unknown substances

EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Tests program

1° group
Control test — test number 1

2° group
Test 1 seed with 1 ml of water and a 1:40 dilution — test number 2,3

3° group
Test 1 seed with 500/1000 ml of water — test number 4,5,6

4° group
Test 1//1/4 seed with 500/1000 ml of water and waiting time of 36 hours
test number 8,9,10

5° group
Test 1//1/4 seed with 500/1000 ml of different drinks
test number 7,11

OPCW Meeting of the SAB TWG
on Sampling and Analysis The Hague,
20/21 NOVEMBER 2008

EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

First results of Test program

Test	Sample type	Sample Treatment	Test Time	Diluition Waiting time	Sample
1	Control test powedesr sampled by swab	/	10.10.2008		**NEGATIVE**
2	Homogenous internal seed pulp unprocessed	1 seed/ 1 ml of H2O (lethal dose for children)	13.10.2008		**POSITIVE**
3	Homogenous internal seed unprocessed	1 seed/ 1 ml of H2O (lethal dose for children)	13.10.2008	Diluition 1:40	**POSITIVE**
4	Homogenous internal seed unprocessed	1 seed/ 500 ml of H2O (lethal dose for children)	17.10.2008		**POSITIVE**
5	Homogenous internal seed unprocessed	1 seed/ 1000 ml of H2O (lethal dose for children)	20.10.2008		**POSITIVE**
6	Homogenous internal seed unprocessed	1/4 seed/ 1000 ml of H2O	20.10.2008		**POSITIVE**
7	Homogenous internal seed unprocessed	1/4 seed/ 500 ml of analcolic drink	20.10.2008		**POSITIVE**
8	Homogenous internal seed unprocessed	1 seme/ 500 ml of H2O	21.10.2008	Waiting time 36 hours	**POSITIVE**
9	Homogenous internal seed unprocessed	1/4 seme/ 1000 ml of H20	21.10.2008	Waiting time 24 hours	**POSITIVE**
10	Homogenous internal seed unprocessed	1/4 seed/ 1000 ml of H20	21.10.2008	Diluition 1:40 Waiting time 24 hours	**POSITIVE**
11	Homogenous internal seed unprocessed	1/4 seed/ 1000 ml of analcolic drink	21.10.2008		**POSITIVE**

EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Conclusion

About first tests

- All tests give positive results in all conditions
- High sensivity of biostrip detection also in sample with high diluition conditions
- High sensivity of biostrip detection also in case of quite long waiting time (sample degradation in long time)
- Short time in order to prepare ad process sample (estimated time 20 minutes/sample)
- Possibility to process many sample in short time in order to have a real control of contaminated area

EFFICENCY TESTS ON RICIN TOXIN BIOSTRIPS

Conclusion

About biostreep detection procedures

- Positive results for use of biological laboratory for analysis on-site
- Quite simple procedures to have first detection with biostrips
- Positive results for use of biostrip inside glove box in a biological laboratory for safety operator, is possible to process sample completely inside glove box area - biohazard 3° level- (low level risk)

THANK YOU
FOR
YOUR ATTENTION!

QUESTIONS?