Product Code: 1406

HLA-C single 1.0
Typing Kit

In vitro diagnostics disposal

Instructions Manual

Version 1.6, May 2010

CE

DESENVOLVIMENTO E PRODUÇÃO
DE TESTES DE DIAGNÓSTICO

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Presentation

This kit contains typing plates with dried primers mixes and PCR Master Mix for low resolution typing of HLA-C gene.

Product Changes and Improvements

The HLA-ABC Box specificity and interpretation tables are constantly updated, to include new HLA class I alleles described. This product can also be improved in order to increase the yield of the specific PCR product.

The primers exchanged, added or modified, compared to the previous lot, are detailed in the table below.

Tube	primers	motivation
N/A		

Quality Control

The specificity of each primer solution of the kit has been tested using 51 DNA samples from the *IHWG Sequence Polymorphism Reference DNA SSOP Panel* (see cell line validation sheet).

No false positive or negative amplifications were obtained.

The negative control tube can detect cross-contamination with PCR products.

Cell line validation sheet

HLA-C low resolution typing kit			
Cell line		Cellular typing HLA-Cw*	HLA-C Positive wells
9215	M7	0401	6
9273	LADA	0701;0802	10/14
9263	G085	12022;1502	15/16/19
9373	FH1	0701;0802	10/14
9030	JHAF	1502	19
9035	JBush	1203	17
9045	TUBO	0704;1502	13/19
9220	XLI-ND	0602;0801	8/14
9077	T7527	0102	1
9085	EJ32B	0501	7
9103	KT14	0801;1402	14/18
9374	FH2	0501;0304	7/4
9375	FH3	0401;0802	6/14
9364	GRC202	03041;04011	4/6
9371	ISH4	04011;0102	1/6
9368	280599	0702/3	11
9367	LCK	0702;1202	11/15/16
9394	BPOT	03031	13
9048	LBUF	0602	8
9032	BSM	0304	4
9237	APA	0801;1203	14/17
9253	THAI742	0102;03031	1/3
9369	ISH3	04011	6
9380	FH6	02022;1505	9/22
9376	FH4	02022	2
9266	PAR	03041;0801	4/14
9377	FH5	0102;1601	1/23
9068	BM9	0401	6
9056	K0SE	1203	17
9009	KAS011	0602	8
9381	FH7	0702;0501	7/11
9385	FH11	0702;1601	11/23
9047	PLH	0602	8
9392	GN00218	0602;0701	8/10
9040	BM15	0701	10
9092	BM92	0102	1
9370	230699	0702;1402	11/18
9372	ISH5	0803;0102	1/14
9052	DBB	0602	8
9267	LE023	0102;15051	1/22
9382	FH8	0302;0102	1/4/5
9366	Daudi	0302;0602	4/5/8
9014	MGAR	0701	10
9053	HOR	1403	18
9021	RSH	1701	25
9024	KT17	0303;0401	3/6
9016	RML	1502	19
9297	HAG	1701	25
9386	FH12	0501;02022	2/7
9387	FH13	0401;03042	6/4

HLA-C Box 1.0 Typing Kit Components

- **HLA-C typing plate**[+] (36 typings)

  12 plates (3 samples each) (Keep at -30 / -15 ºC)

- **PCR Master Mix (With Taq DNA Polymerase)**

  12 X 310 µl (keep at -30 / -15ºC)

- **PCR plate sealers**

  12 plate sealers

- **Instructions Manual**

  1 Instructions Manual

[+] With dried specific primers pares (25 primer pairs)

PCR Master Mix Components

Nucleotides
Final concentration of each dNTP: 600 µM

PCR Buffer
Final concentration: 3,3x NH_4 Buffer; 2,0 mM $MgCl_2$ and 0,4 U/µl Taq DNA polymerase, pH 8.3.

Glycerol
Final concentration: 16,6%

Cresol Red
Final concentration: 300µg/ml

PCR amplification protocol

Reagents

- DNA Sample (100-200 ng/µl)
- PCR Master Mix
- $_{dd}H_2O$ (not supplied)

DNA Extraction

For SSP typing highly pure DNA is needed. We recommend isolation of DNA using any extraction kit with CE marking, which guarantees an OD ratio 260/280 higher than 1.6 and a 100ng – 200 ng/µl DNA concentration.
Alternatively, the DNA can be extracted using trimethyammoium-bromide salts (DTAB/CTAB) or by salting out, dissolving it in TE Buffer. The same OD and concentration values should be assured.
DO NOT USE HEPARINISED BLOOD WITH THIS METHOD

PCR Amplification

1. Spin briefly the DNA and Master Mix tubes.
2. Add:
   - **102 µl of PCR Master Mix,**
   - **205 µl of ddH_2O, and**

   to a 0,7 ml or 1,5 ml tube.
3. Vortex the tube vigorously for 15s.
4. Load **10 µl** of the mix into the negative control well.
5. Add **27 µl of DNA sample (conc. 100-200 ng / µl)** to the PCR mix tube.
6. Vortex the tube vigorously for 15s.
7. Load **10 µl** of the mix into each specific plate wells (26 specific primers).

8. Repeat the previous steeps for other 2 DNA sample to complete the HLA-C typing plate.
9. Close the typing plate with a self-adhesive lid and put it in a 96 well thermo cycler.

PCR Cycling Parameters

Step	Temperature	time	Cycle
Denaturation	96 ºC	1 min	1
Denaturation Annealing Extension	96 ºC 70 ºC 72 ºC	25 sec 45 sec 30 sec	5
Denaturation Annealing Extension	96 ºC 65 ºC 72 ºC	25 sec 45 sec 30 sec	21
Denaturation Annealing Extension	96 ºC 55 ºC 72 ºC	25 sec 1 min 2 min	4
Extension	72 ºC	10 min	1
Keep (optional)	4 ºC	Infinite	1

10. Keep the plate at 2-8 ºC after the PCR have finished.
11. Detect the PCR products with 2% agarose gel electrophoresis.

Gel Electrophoresis protocol

PREPARING 2% AGAROSE GEL

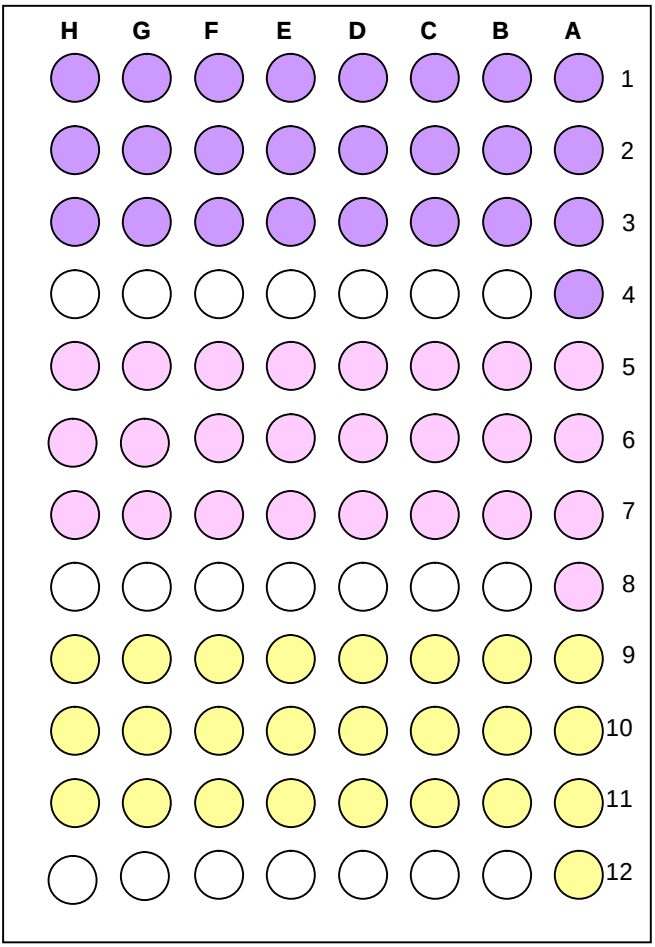
1. Dissolve **4 grams** of electrophoresis **grade agarose** powder in **200 ml** of **1X TAE buffer**.
2. Melt the agarose powder completely in a microwave oven.
3. Cool the heated agarose gel to **~ 50°C.**
4. Add at least 10 µl of **ethidium bromide**[++] (10 mg/ml) or **Sybr Safe**[TM] (100000 x concentrate) to the heated agarose. Stir until it is thoroughly incorporated.
5. On a balanced surface, set up a gel plate with **96 wells**.
6. Cast a **5mm** thick gel on the plate.
7. Allow the gel to settle.

[++]**Caution**, this reagent is a strong mutagenic agent (read carefully its MSDS before using it).

GEL ELECTROPHORESIS

1. Submerge the gel in 1X TAE buffer in a gel box.
2. Gently remove the caps to avoid splashing of PCR products.
3. Load 10 µl into each well on the gel.
4. Connect the electric leads and turn on the power supply (115V). Electrophoresis for ~ 20 minutes, or until 2/3 of the lane.
5. Transfer the gel onto a UV transilluminator, document the result by photography.
6. Use the ***result interpretation sheet*** (1-4) to interpret results.

HLA-C single 1.0 plate



HLA-C single 1.0 Plate Identification

Position			HLA
1a	5a	9a	C
1b	5b	9b	C
1c	5c	9c	C
1d	5d	9d	C
1e	5e	9e	C
1f	5f	9f	C
1g	5g	9g	C
1h	5h	9h	C
2a	6a	10a	C
2b	6b	10b	C
2c	6c	10c	C
2d	6d	10d	C
2e	6e	10e	C
2f	6f	10f	C
2g	6g	10g	C
2h	6h	10h	C
3a	7a	11a	C
3b	7b	11b	C
3c	7c	11c	C
3d	7d	11d	C
3e	7e	11e	C
3f	7f	11f	C
3g	7g	11g	C
3h	7h	11h	C
4a	8a	12a	C
4b	8b	12b	Negative Control
4c	8c	12c	Positive Control
4d	8d	12d	Empty wells
4e	8e	12e	
4f	8f	12f	
4g	8g	12g	
4h	8h	12h	

Results Interpretation sheet (1/1)

Well			HLA	Allele	Serotype	ampl	contr **
1a	5a	9a	Cw	Cw*0102	**Cw*01**	1026	1600+796
1b	5b	9b	Cw	Cw*0202,*1701/2	**Cw*02; 17**	521	1600+796
1c	5c	9c	Cw	Cw*0303	**Cw*03**	530	1600+796
1d	5d	9d	Cw	Cw*0302/4	**Cw*03**	529	1600+796
1e	5e	9e	Cw	Cw*0302	**Cw*03**	206	1600+796
1f	5f	9f	Cw	Cw*0401-3, *1801	**Cw*04; 18**	330	1600+796
1g	5g	9g	Cw	Cw*0501	**Cw*05**	563	1600+796
1h	5h	9h	Cw	Cw*0602	**Cw*06**	304	1600+796
2a	6a	10a	Cw	Cw*1801	**Cw*18**	500	1600+796
2b	6b	10b	Cw	Cw*0701	**Cw*07**	516	1600+796
2c	6c	10c	Cw	Cw*0702/3	**Cw*07**	302	1600+796
2d	6d	10d	Cw	Cw*0703, A*2604	**Cw*07 A*26**	494	1600+796
2e	6e	10e	Cw	Cw*0704	**Cw*07**	563	1600+796
2f	6f	10f	Cw	single 162bp=Cw*0802, 162&632bp=Cw*0801/3	**Cw*08**		1600+796
2g	6g	10g	Cw	Cw*1202,*1301	**Cw*12; 13**	449	1600+796
2h	6h	10h	Cw	Cw*1202	**Cw*12**	537	1600+796
3a	7a	11a	Cw	Cw*1203	**Cw*12**	453	1600+796
3b	7b	11b	Cw	Cw*1402/3	**Cw*14**	541	1600+796
3c	7c	11c	Cw	Cw*1502	**Cw*15**	407	1600+796
3d	7d	11d	Cw	Cw*1503	**Cw*15**	406	1600+796
3e	7e	11e	Cw	Cw*1504,*0701	**Cw*15; 07**	423	1600+796
3f	7f	11f	Cw	Cw*1505	**Cw*15**	512	1600+796
3g	7g	11g	Cw	Cw*1601	**Cw*16**	424	1600+796
3h	7h	11h	Cw	Cw*1602	**Cw*16**	475	1600+796
4a	8a	12a	Cw	Cw*1701/2	**Cw*17**	512	1600+796
4b	8b	12b	Positive Control				1600+796
4c	8c	12c	Negative control				
4d	8d	12d	Empty wells				
4e	8e	12e					
4f	8f	12f					
4g	8g	12g					
4h	8h	12h					
DNA 1	DNA 2	DNA 3					

****Control primer pares match with non-allelic sequences. The internal positive control primer pairs amplify segments of the HLA-DRB1 gene and adenamoutous polyposis coli (PIC1) gene. Giving rise to 1600 + 796 base pair fragments and 256 base pair fragment respectively.**

In the presence of the specific band amplification the control band intensity often decreases.

The PCR reaction is only valid in the presence of control band or, in some cases, in the presence of the specific band. In the absence of the control band, please repeat the typing.

Results Interpretation Table (1/1)

Well No.	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95
Specific Band	1026	521	530	529	206	330	563	304	500	516	302	494	563	162	449	537	453	541	407	406	423	424	512	512	475
Cw*01	+																								
Cw*02		+																							
Cw*03			*	*	*																				
Cw*04						+																			
Cw*05							+																		
Cw*06								+																	
Cw*07										+	*	*	*								*				
Cw*08						+								+											
Cw*12															+	*	*								
Cw*13															+										
Cw*14																		+							
Cw*15																			*	*	*	*			
Cw*16																							*	*	
Cw*17		+																							+
Cw*18									+																
A*2604												+													

* Positive for some subtypes

Troubleshooting Guide

PROBLEMS	POSSIBLE CAUSES	SUGGESTIONS
The control and specific bands are weak.	Concentration of DNA sample is too low.	Check DNA quality and concentration
		Re-extract the DNA sample or try not add water into the PCR Mix
		Repeat typing with a good quality DNA sample
	DNA polymerase inhibitors in the DNA sample	Re-purify the sample DNA
		Repeat typing with a good quality DNA sample
Missing internal control bands in one or several lanes.	DNA polymerase inhibitors in the DNA sample.	Re-purify the sample DNA
		Repeat typing with a good quality DNA sample
	Dried PCR amplification products	Check the plate sealing
		Repeat the typing using a PCR MicroMat and/or overlay the PCR reaction mix with mineral oil
False negative of a specific band while the internal control appears normal	Degradation of DNA sample	Re-extract the DNA sample with fresh material
		Repeat typing with a good quality DNA sample
More than two specific alleles are detected/ Ambiguous results	Excess of template DNA	Check DNA quality and concentration
		Dissolve the DNA sample in $_{dd}$H2O in order to have the proper concentration
		Repeat typing with a good quality DNA sample
	Contamination with previously amplified PCR products or with other DNA samples during the DNA extraction or PCR preparation steps	Clean the working area
		Work in separated pre-PCR and post-PCR rooms
		Keep different lab coats in pre-PCR and post-PCR rooms
		Change protective gloves frequently
		Repeat typing with a good quality DNA sample
Blurred bands	Degradation of DNA sample	Re-extract the DNA sample with fresh material
		Repeat typing with a good quality DNA sample
	Excess of template DNA	Check DNA quality and concentration
		Dissolve the DNA sample in $_{dd}$H2O in order to have the proper concentration
		Repeat typing with a good quality DNA sample
	Electrophoresis Buffer Problems: wrong buffer or older buffer	Use a fresh recommended buffer

Precautions and Warnings

PCR amplification allows the amplification of small quantities of sample DNA in an exponential way. However, this is also true for foreign DNA, which can contaminate our PCR method. Consequently, special laboratory practices are necessary in order to avoid false positive amplifications. Bellow is listed Genebox recommendations to circumvent contaminations:

- Work in separated pre-PCR and post-PCR rooms.

- Laboratory workflow must be unidirectional, from pre-PCR to post-PCR area.

- Specific equipment for each working area must be used (sample preparation, amplification and preamplification).

- All equipment used in post-PCR should not leave this area.

- Use dedicated micropipettes, gloves and lab coats in each area.

- Use non talcum powder gloves (since talcum could inhibit the PCR reaction).

- Use filter tips in order to avoid cross contamination.

- Check regularly micropipettes, in order to ensure that they are accurate within 5 % of fixed volume.

- Use different micropipettes depending on the volume we wish to load.

- Check regularly thermocyclers, in order to ensure that they are accurate within 1% of fixed temperature.

- Open and close reagent vials carefully. After use, close vials and store at indicated temperatures.

- Do not use a kit after its expiration date.

- packaging material included within the kit is resistant to the indicated storage conditions. Storage at different conditions can cause breakage of the material, and possible contamination of the kit reagents.

- plastic material included within the kit is resistant under normal conditions of use. Use of plastic material in extreme conditions may cause its breakage, and therefore, the impossibility to use the kit.

- check suitability of DNA quantity and quality before use the kit.

General instructions for laboratory safety:

- do not eat, drink or smoke in laboratory work areas

- wear disposable gloves

- wear clean lab coats and eye protection

- wash hands thoroughly after handling specimens and test reagents

- clean the working area before and after kit handling.

Technical Guide

1. DNA Quality and Concentration

For optimal results with the HLA-C single 1.0 Typing Kit™ the quality of DNA is critical. Good quality DNA means an OD ratio 260/280 higher than 1.6 and the major portion of DNA should run higher than 9.4 kb on an agarose gel. Different quality and concentration values require DNA re-extraction.
The quantity of DNA should be 100ng – 200 ng/µl. Excess of DNA can cause unspecific amplification.
We recommend any DNA extraction kit which has CE marking, in order to obtain this highly DNA purity

2. Taq Polymerase

HLA-C single1.0 Typing Kit™ kits have been intensively tested with the Taq DNA Reagente 5 (Reagente 5, Lisbo, Portugal).

3. PCR Master Mix

For optimal results with the HLA-C single 1.0 Typing Kit™ the use of the master mix supplied is obligatory.

4. Amplification Procedure

At the end of PCR, examine the degree of evaporation and condensation of PCR reaction mixture. If there is more than 20% volume loss do not validate the results. In order to prevent this you should overlay the PCR reaction mixture with mineral oil or use a MicroMat. It is also a good practice to maintain QC records on the heating lid.
If the temperature of the heating lid is not high enough, it will cause condensation problems on the lid.

5. Thermal Cycler

We recommend the use of any thermocycler with the following characteristics:
- heating rate up to 2.5ºC/sec; cooling rate up to 1.5ºC/sec; temperature range 4-100ºC; temperature uniformity ±0.5ºC; heated lid up to 100ºC.

6. Validity

As specified in the package

If your problems persist, do not hesitate to contact our technical support

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Guarantee

geneBOX - R&D Diagnostic Tests guarantees that the primers in HLA-ABC Box typing kit have the specificities given in the Results Interpretations Sheet/Tables of the product insert.

1. Typing plate

When stored at -20ºC, the dried primers are stable for 12 to 19 months from the date of manufacture (see lot validity in the package).
When stored at 4ºC, the dried primers are stable for 12 months from the date of manufacture (see lot validity in the package).
At room temperature, the dried primers are stable for 3 to 4 weeks from the date of the reception.
When the sealer is removed the dried primers steal be stable for 2 days, maximum, in dried conditions.

2. PCR Master Mix

When stored at -20ºC, the PCR Master Mix is stable for 18 months from the date of manufacture (see lot validity in the package).
When stored at 4ºC, the Master mix is stable for 15 days from the date of the reception.
At room temperature, the master mix steal be stable for 3 days from the date of the reception.
The master mix should not be left or stored with the cap open.

3. DNA

Using extracted DNA from salting out or any kit procedure the samples should be stored at 4ºC or -20ºC. If you chose to freeze the samples you must avoid repeated cycles of heating/freezing, in order to preserve your sample stability.
The DNA samples stored in dH_2O are stable for at least 2 to 4 weeks (at 4ºC) or 24 months (at -20ºC).
The DNA samples stored in buffer are stable for at least 12 months (at 4ºC) or 5 years (at -20ºC).

Warranty

geneBOX - R&D Diagnostic Tests warrants its products to the client against defects in materials and contents under normal application. The company products under this warranty shall be replaced, at no charge, to the damaged client.

This warranty applies only to products that have been handled and stored in accordance with its recommendations/specifications.

The claims must be posted directly to geneBOX in writing and must be accompanied by a copy of the purchaser's invoice.

This product may not be reformulated, repacked or resold in any form without geneBOX - R&D Diagnostic Tests consent.

Declaration of conformity

Product Name: HLA-C single

Product Number: GB.14.06

Intended use: HLA-C low resolution histocompatibility testing.

Manufacturer:

geneBOX - R&D Diagnostic Tests,
Biocant – centro de inovação em biotecnologia
núcleo 4, lote 3
3060-197 Cantanhede,
Portugal

We, geneBOX - R&D Diagnostic Tests, hereby declare that this product, to which this declaration of conformity relates, is in conformity with the following standards and other normative documents ISO 9001:2008 and ISO 13485:2003, following the provisions of the 98/79/EC Directive on *in vitro* diagnostic medical devices as transposed into the national laws of the Member States of the European Union.

The technical file of the product is maintained at geneBOX - R&D Diagnostic Tests, Biocant Park, Parque tecnológico de Cantanhede, 3060-197 Cantanhede, Portugal.

Sandra Balseiro
Technical Director

Material Safety Data Sheet (MSDS) (1/3)

geneBOX - R&D Diagnostic Tests™ PCR-SSP Kits

geneBOX ™ PCR-SSP typing products

This Material Safety Data Sheet (MSDS) applies to all geneBOX - R&D Diagnostic Tests SSP™ typing kits

1. Chemical products and company identification

Date of Issue:	May 2010
Product group:	geneBOX™ PCR-SSP Typing Products
Manufacturer:	geneBOX - R&D Diagnostic Tests, biocant – centro de inovação em biotecnologia núcleo 4, lote 3 3060-197 cantanhede, portugal
tel/fax:	+351 231 410 946/ +351 231 410 947
e-mail:	info@genebox.com

2. Composition and reagents information

Component	Chemical	Common Name
Plate	Deoxyribonucleic acid Cresol Red	Oligonucleotide
PCR Master Mix	Deoxyribonucleotides NH_4 Buffer Magnesium chloride Cresol Red Glycerol	Nucleotides MgCl2 Glycerine

3. Physic-chemical properties:

Components	Appearance	Colour	Odour
Plate	dried, in plate wells	Red	none
Master Mix	liquid	Pink/red	none

4. Toxicological information

Chemical	Toxicities
Glycerol	LD50= oral 4090 mg/kg (mouse) LD50= oral 12600 mg/kg (rat) LD50= oral 1480 mg/kg (human)

5. Stability and reactivity

Conditions to avoid: Heat and moisture.
Incompatibilities: Strong oxidizing agents, strong bases.

Material Safety Data Sheet (MSDS) (2/3)

6. Personal protection.

Hand protection: Wear appropriate chemically resistant gloves.
Eye protection: Chemical safety goggles are recommended.
Skin protection: Wear laboratory coat.

7. Handling and storage

Handling: Avoid substance contact.
Storage: Protect from light. Store at temperature indicated on package.
Package Damage: reject damaged components.

8. Hazards

Master Mix Components: may be harmful by **inhalation, ingestion or skin absorption**. May cause eye and skin irritation. Material is irritating to mucous membranes and upper respiratory tract. **Ingestion** of large amounts can cause stomach pains, vomiting or diarrhoea.

9. First aid measures

In case of eye contact: Immediately flush eyes with large amounts of water for at least 15 minutes. Call a physician.
In case of skin contact: Immediately wash skin with soap and large amounts of water. Wash contaminated clothing before re-use.
In case of ingestion: Wash out mouth with water provided person is conscious. Call a physician if needed.
In case of inhalation: remove to fresh air, if not breathing give artificial respiration. If breathing difficult, give oxygen. Call a physician.

10. Fire fighting measures

Extinguishing media: Water, carbon dioxide, dry chemical powder or appropriate foam.
Extinguishing media NOT to use: None are known.
Special exposure hazards: May emit toxic fumes of carbon dioxide, carbon monoxide, nitrogen, phosphorus, hydrogen chloride, and hydrogen gas under fire conditions.
Special fire-fighting equipment: When large amounts of substances are released work only with self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.

11. Accidental release measures

Personal Precautions: Avoid substance contact. No further requirements.
Cleaning Method: Clean up affected area. No further requirements.

Material Safety Data Sheet (MSDS) (2/3)

12. Ecological information
No data available.

13. Waste disposal information
Waste disposes in accordance with all applicable regulations (the disposals should be incinerated).

14. Transport information
During transportation the temperature could not exceed 25ºC.
Transportation should not exceed 3 days.

15. Other information
The above information is based on our current level of knowledge, but does not purport to be all-inclusive and shall be used only as a guide. *geneBOX - R&D Diagnostic Tests* shall not be held liable for any damage resulting from handling or from contact with the above products.

If your problems persist, do not hesitate to contact our technical support

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References

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geneBOX
DESENVOLVIMENTO E PRODUÇÃO DE TESTES DE DIAGNÓSTICO

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