Global Polio Laboratory Network

Guidance Paper 4

Cell Authentication Testing

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Global Polio Laboratory Network

1. SCOPE

This document is intended to describe the test for authentication of cell lines, designed by National Institute for Biological Controls and Standrads (NIBSC), which is used in GPLN laboratories to detect cell line cross-contamination. The accuracy of poliovirus surveillance strongly relies on the quality of cell lines used for virus isolation. A combination of infections in L20B and RD cells, including cross passage between them, is used to ensure maximum sensitivity and specificity for poliovirus isolation. Contamination of cell lines can occur due to poor handling techniques such as sub-culturing different cell lines at the same time, using the same media for different cell lines, incomplete cleaning of equipment/cabinets, mislabelling of cell bank tubes, etc. Cell cross contaminations are usually not identifiable by microscope observation. Using mixed cell cultures can lead to wrong result interpretation (Fig. 1) which could cause additional work and unnecessary alarm and eventually failing to detect poliovirus in samples.

	RD	L20B	RD cont. with L20B	L20B cont. with RD	RD swap with L20B	L20B swap with RD
PV	+	+	+	+	+	+
NPEV	+	-	+	+	-	+
PV + NPEV	+	+	+	+	+	+
Negative	-	-	-	-	-	-

Figure 1. Outcome of cell line cross contamination. Green, no change in final interpretation. Orange, partial change. Red, critical change.

2. OBJECTIVES

The objectives of this document are to :

- briefly describe the principle for cell authentication (CA) testing
- help identifying situations when cell authentication testing is required
- describe test format
- give guidance on sample preparation for CA testing
- describe interpretation of results and reporting
- define a follow-up action plan
- assist on implementation of real-time PCR CA test and Quality Assurance in GPLN labs.

3. Test principle

The CA test is based on the detection of the mitochondrial Cytochrome C Oxidase Subunit 1 (CO1) gene by real-time PCR. The nucleotide sequence of the CO1 gene is specific for different animal species so it is highly conserved within-species and highly diverse between-species. The CO1 gene is flanked by conserved regions between species making it a convenient target for barcoding analysis. This region is 648 nucleotide base pairs long and varies less than 1-2% among individuals of the same species and several % for closely related species. By using the differences in CO1 sequences it is possible to identify the species derivation of laboratory cell lines and find any contaminants. The CO1 test uses primers with human or mouse DNA

sequences in two separate reactions. The test is very specific and sensitive as it can detect mitochondrial DNA corresponding to 0.01 human cells (muscle fibroblast cells like RD cells are known to contain approximately 9,000 mitochondria per cell) (Fig. 2) or to 0.1 mouse cells (L mouse cells are known to contain approximately 1,000 mitochondria per cell).

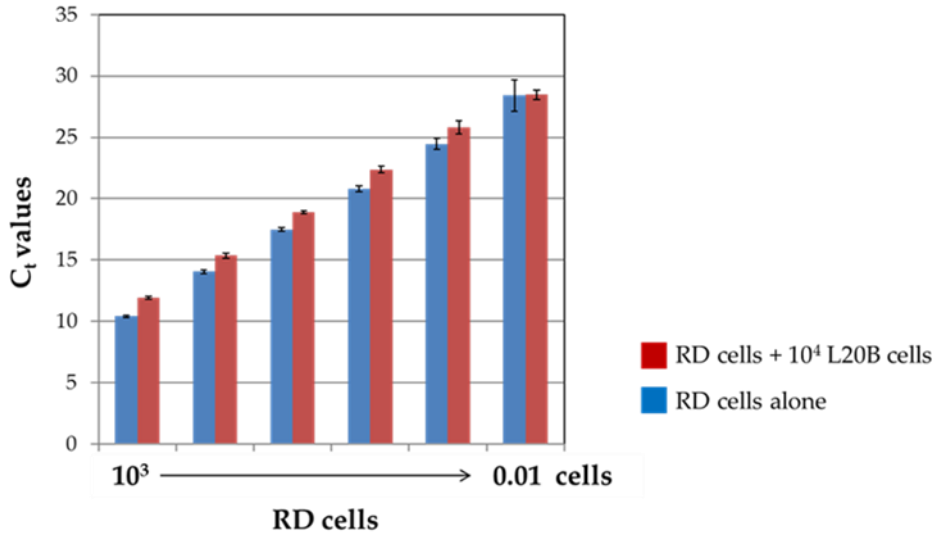

Figure 2. Sensitivity and specificity of CA test. Real-time PCR analysis of DNA from serial dilutions of RD cells alone or in a background of 104 L20B cells using human primers. The corresponding numbers of cells are indicated.

CO1 universal primers can also be used in combination with DNA melting curve analysis that measures the temperature at which the double strand PCR product dissociates, which is dependent on the nucleotide sequence, to test for the presence of cells from other origin: rabbit, dog, non-human primates, etc. that might be used in the lab for other purposes (Fig. 3).

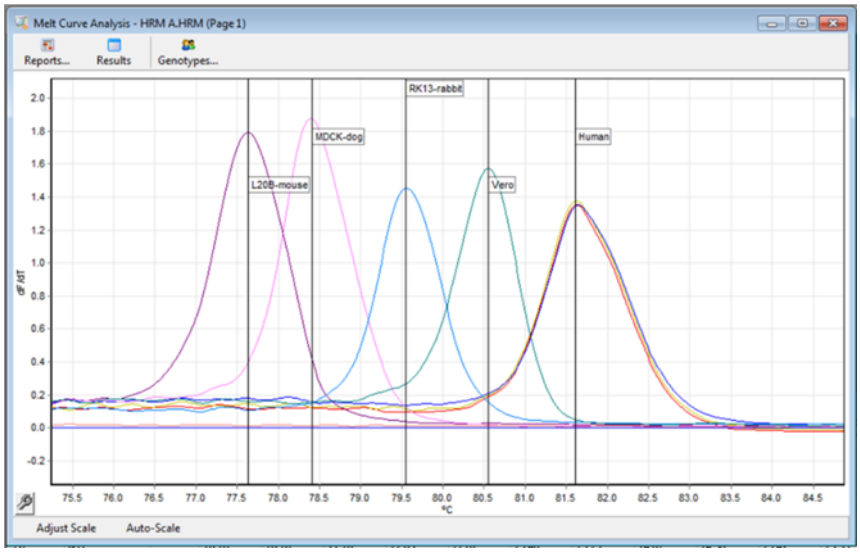

Figure 3. Universal CA test. Real-time PCR analysis of DNA from cell lines from different animal species using universal CO1 primers. Melting DNA curves for each cell line are shown.

Location of CO1 primers in the genes is shown in Fig. 4.

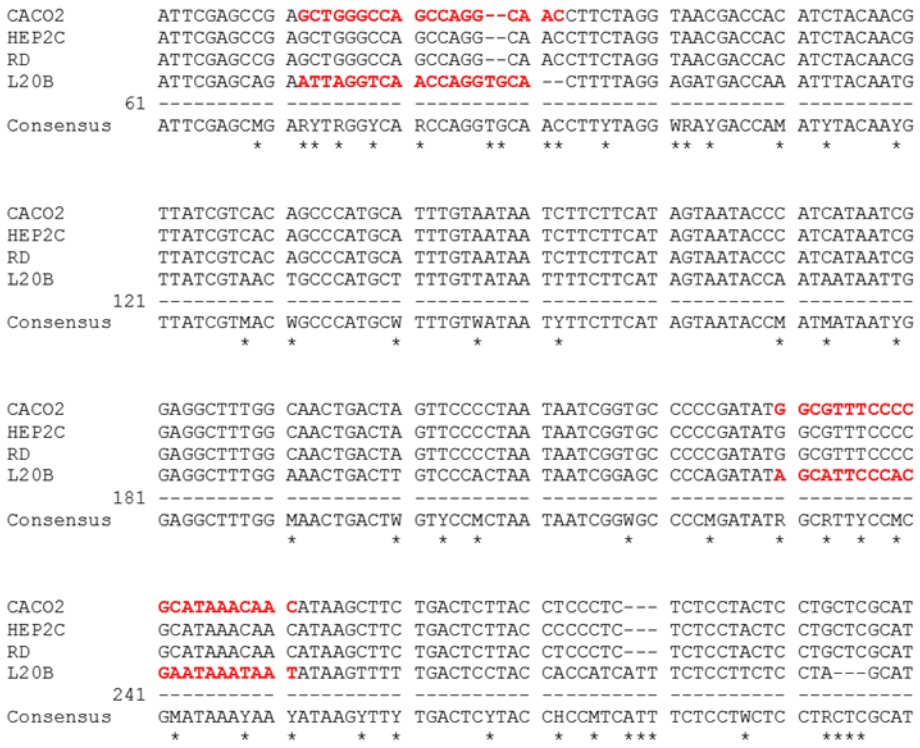

Figure 4. Location of CA primers. Location of human (top sequence) and mouse (bottom sequence) primers used in CA real-time PCR tests are shown in red.

4. Frequency of testing - Situations when to suspect cell lines cross-contamination

- Master cell banks, working cell banks and cell banks for distribution in laboratories supplying cells to the GPLN should be tested as soon as they are established.
- Cell banks at all GPLN laboratories should be tested if there is suspicion of cell-line cross contamination:
  - Sudden and significant increase in NPEV isolations in L20B cells
    - NPEV in ITD
    - Virus not neutralized with anti-PV sera
  - Sudden and significant drop in NPEV isolations
  - Failed PT due to:
    - False L20B + result in sample containing NPEV only: possible contamination/swap of L20B cells with RD cells
    - False RD - result in sample containing NPEV only: possible contamination/swap of RD cells with L20B cells
  - Atypical/Inconclusive CPE result:
    - No CPE progress to +3 (particularly after second passage)
    - Cells “recovering” from CPE
  - Changes in cell sensitivity (higher titres in L20B cell lines)

5. Test format and Quality control

- Instructions for the preparation of DNA samples and shipment to the testing laboratory are given in the Appendix below
- Standard preparations containing serial dilutions of DNA from RD or L20B cells are used to establish a standard curve and produce quantitative results of test samples.
- Samples containing DNA mixtures from RD and L20B cells are used as controls in every test.
- The test samples are analysed in duplicate in two independent assays for confirmation.

- Primer combinations:
  - Human forward + Human reverse
  - Mouse forward + Mouse reverse
  - Universal primers

Amplification conditions are shown in Fig. 5 (Rotorgene). These would need to be adapted to be used in other real-time PCR platforms.

Hold : 95°C for 15 min	
Cycling (35 repeats)	Step 1 : 94°C for 15 secs
	Step 2 : 55°C for 20 secs
	Step 3 : 72°C for 20 secs, (acquire to Sybr Green channel)
Melt (72-95°C) , Rising by 1degree, hold 90 secs on the 1st step, hold 5 secs on next steps (acquire to Green channel)	

6. Test results, report and interpretation

- Test validity based on results for control samples
- Test sensitivity based on results with reference sample dilutions
- Test result for laboratory cell samples classified as satisfactory or contaminated
- Quantification of specific DNA content based on standard curves and Ct values can give an indication on level of contamination
- Combination of results with human and mouse primers will determine whether there is contamination or cell line swapping
- Full report is prepared and shared with lab and Regional Coordinator (example in Fig. 6)

No.	Colour	Name	Type	Given Conc (cell/reaction)	Ct	Calc Conc (cell/reaction)	Result	Outcome
1		L20B S1	Standard	12500	13.46	10200	Positive	Satisfactory
2		L20B S2	Standard	1250	16.86	1346	Positive	Satisfactory
3		L20B S3	Standard	125	20.36	167	Positive	Satisfactory
4		L20B S4	Standard	12.5	24.69	12	Positive	Satisfactory
5		L20B S5	Standard	1.25	28.85	1	Positive	Satisfactory
6		[L20B+RD] C1	Control		17.33	1012	Positive	Satisfactory
7		[L20B+RD] C2	Control		21.26	97	Positive	Satisfactory
8		[L20B+RD] C3	Control		24.96	10	Positive	Satisfactory
9		RD C7	Control				Negative	Satisfactory
10		L20B C8	Control		12.80	15160	Positive	Satisfactory
11		L20B p17+4 (2009.12.2)	Unknown		15.35	3307	Positive	Satisfactory
12		L20B p17+4 (2009.12.2)	Unknown		15.20	3620	Positive	Satisfactory
13		L20B p21+2 (2011.6.1)	Unknown		14.82	4534	Positive	Satisfactory
14		L20B p21+2 (2011.6.1)	Unknown		14.73	4793	Positive	Satisfactory
15		L20B p21+5 (2013.12.27)	Unknown		14.37	5945	Positive	Satisfactory
16		L20B p21+5 (2013.12.27)	Unknown		14.43	5716	Positive	Satisfactory
17		RD p227+4 (2009.11.20)	Unknown				Negative	Satisfactory
18		RD p227+4 (2009.11.20)	Unknown				Negative	Satisfactory
19		RD p229+2 (2011.6.1)	Unknown				Negative	Satisfactory
20		RD p229+2 (2011.6.1)	Unknown				Negative	Satisfactory
21		RD p229+6 (2013.12.27)	Unknown				Negative	Satisfactory
22		RD p229+6 (2013.12.27)	Unknown				Negative	Satisfactory
23		NTC*	NTC				Negative	Satisfactory

*NTC= No Template Control

Figure 7. Example of CA report.

7. Follow-up action plan

If contamination is identified action depends on when contamination might have occurred: master, working, passaged cell samples. Master and working cell banks must be tested, discarded and changed if found to be contaminated. Cell aliquots from previous cell sensitivity assays should be tested if available to try to determine when contamination occurred. Recent results for virus isolation/ITD, cell sensitivity and virus isolation panels should be reviewed to evaluate the possible impact and need for re-testing.

8. Plan for implementation of CA rtPCR testing in GPLN laboratories

NIBSC can design implementation plans for laboratories willing to adopt the technique. These will require adaptation of the CA test to different real-time PCR platforms. Samples to assist implementation can be provided.

APPENDIX. Sample application onto FTA Micro Cards for Cell Authentication

(Adapted from US CDC Protocol for Poliovirus isolates)

I. Materials:

- Indicating FTA Micro cards (Whatman WB120211)
- Multibarrier Pouch (Whatman WB100036)
- Plastic Ziploc Minigrip Bags, 4 x 6" (Whatman WB10548232)
- Desiccant Packs (Whatman 10548234)
- Microcentrifuge or cryovial Tube, 1.5-1.8 ml, sterile
- Lab marker, fine point
- Biohazard waste container.
- Micropipette (200 µl)
- Pipette tips, filtered (200 µl)
- Lab coat
- Gloves
- Laboratory bench liners
- Timer or Clock
- RD and L20B cell lines to be spotted

II. Method: Prepare each cell line sample one at a time. Use one FTA card for each sample

1) Label each FTA card with the (1) Name of cell line (2) Passage number; (3) date of application, and (4) Card No..

   Important: Wear gloves when handling FTA cards to avoid cross-contamination with your own DNA.

2) Label one tube for each cell line to be spotted.
3) In a biosafety cabinet, transfer 200µl cell suspension containing 1 x 10^6 cells per ml into the labeled tube.
4) **THEN EITHER**:

   Freeze the cell suspension at -20 °C (or lower) and thaw at room temperature. Vortex mix and centrifuge the cell lysate at 12,000 g at 4°C for 10 minutes to pellet cell debris. Then apply the supernatant to the FTA card (step 5).

   OR add sufficient cell suspension containing 2 x 10^5 cells directly to the FTA card (step 5).

There is no need to remove DMSO, this will be removed during the DNA extraction process.

5) In a biosafety cabinet apply 200 µl of the cell supernatant or suspension in a concentric circular motion. (MAKE SURE TO EVENLY DISTRIBUTE THE SAMPLE ACROSS THE CIRCLE)
6) Allow the card to dry in the biosafety cabinet at room temperature, then apply a further 200µl to the card and allow the card to dry completely. It may take up to one hour or more, depending on the temperature and humidity in the laboratory.
7) Place each card in a labeled multi-barrier pouch with a desiccant and place in a clean dry zip lock bag. To avoid cross-contamination, use one plastic zip-lock bag per sample.
8) Store the cards at -20°C (this is the ideal storage conditions) until shipped.
9) Prepare a line listing of the cell lines being submitted for cell authentication (See Sec. V.) and include in the shipment.
10) Ship cards in regular shipment as a "Category B Biological Substances" at room temperature via EMS or courier service (FedEx Letter Pak, DHL or another shipper).

III. Notes:

- Before handling FTA cards, thoroughly clean the work surface area.
- Always handle FTA cards with gloved hands to prevent cross-contamination with your own DNA.
- Do not store cards at -20°C or 4°C for longer than 5 business days before shipping.
- Make sure the biosafety cabinet is left running while drying the cards.
- Follow WHO's Standard Operating Procedure for decontamination and discard of infectious materials.
- Each card should be in a separate bag/pouch with a desiccant in each bag/pouch.
- Make sure card does not get stuck in the seal of the pouch.
- MAKE SURE THAT THE CARDS ARE COMPLETELY DRY BEFORE SHIPPING!