Appendix 1

Poliovirus rRT-PCR ITD 4.1 Addendum Kit

The ITD 4.1 addendum is a kit to supplement the ITD 4.0 kit. The ITD 4.1 provides two new reaction assays, WPV1-2015 and AFR WPV3-2015. The new assays are direct replacements for Duplex WPV1 and AFR WPV3. **Please discard the Duplex WPV1 and AFR WPV3 assay tubes from the ITD 4.0 kit.**

The replacement of the Duplex WPV1 and AFR WPV3 with the WPV1-2015 and AFR WPV3-2015, respectively, constitutes the new ITD 4.1 kit. In addition to the replacement of assays, the ITD 4.1 also runs under a more stringent reaction condition. The ITD 4.1 has been verified to work with Quanta Biosciences qScript XLT One-Step RT-qPCR ToughMix (CAT#95132-500). The identification of the real-time reaction mix is for the convenience of the end user and does not constitute an endorsement of the product.

Please follow the instructions as provided in the ITD 4.0 insert with the following exception:

7) Place strips in real-time thermocycler and cycle as shown below. If using a thermocycler with a rapid ramp speed, program the ramp from **50°C to 72°C for 45 sec** (note for the ABI 7500, you can use 25% ramp speed between the anneal and extension temperatures for all assays). Thermocyclers with regular ramp speeds can use the default ramp time; Stratagene Mx3000P and similar machines do not have adjustable ramp capabilities. An additional intermediate step between the lower and higher temperature in the PCR cycle compensates for the inability to adjust the ramp time between anneal and extension):

a) RT reaction, **50°C for 30 minutes**

b) Inactivate RT, **95°C for 1 minute**

c) PCR cycles (all primer sets):

Using an ABI 7500:

95°C for 15 seconds, **50°C for 45 seconds**, then a 25% ramp speed to **72°C for 5 seconds** for 40 cycles. The end point fluorescent data is collected at the end of the 50°C anneal step.

d) Select the appropriate dye filter to correspond with the assay being used [Note: AFR WPV3-2015 uses **CY5** and WPV1 only uses **FAM**]

e) Start run.

	Primers/Probes	Sequence (5'-3')
AFRWPV3-2015	AN919-S	CAGGGAGTAGATGAYCTNAT
	AN920-S	CAGGGGGTTGATGAYTTRAT
	AN924-A	ACKGTGTCTGAYGGNAC
	AN931S-probe	CY5-CNCARAACAGYCTTCCGGATACC-BHQ3
WPV1-2015	AN937-S	GTACAAACCAGTCAYGTNAT
	AN938-S	CGTACAGACTAGRCAYGTNAT
	AN939-A	GAGAATAAYTTGTCYTTKGAYGT
	AN936A-probe	FAM-CATWATGGTTACRCAMGCACCT-BHQ1

Appendix 2: ITD fluorophore changes

	Primers/Probes	Sequence (5'-3')	
AFRWPV3-2015	AN919-S	CAGGGAGTAGATGAYCTNAT	ITD 4.1
	AN920-S	CAGGGGGTTGATGAYTTRAT	
	AN924-A	ACKGTGTCTGAYGGNAC	
	AN931S-probe	CY5-CNCARAACAGYCTTCCGGATACC-BHQ3	
WPV1-2015	AN937-S	GTACAAACCAGTCAYGTNAT	
	AN938-S	CGTACAGACTAGRCAYGTNAT	
	AN939-A	GAGAATAAYTTGTCYTTKGAYGT	
	AN936A-probe	FAM-CATWATGGTTACRCAMGCACCT-BHQ1	

Duplex WPV1 ITD 4.0

AFR WPV1:

1A	GMR AAYARYTTRTCYTTRGA
2S	CRGTICAAACYAGRCAYGTYA
WPV1 Probe-17S	**FAM**-TGYGTRACIATNATGACIGTGGA-**BHQ1**

SOAS WPV1:

1A	ACTGARAAYARYTTRTCYYTKGA
2S	ACRGGRGCYACRAACCCNTT
P1W Probe-20S	**CY5**-CARCAYAGRTCIMGNTCAGA-**BHQ3**

AFR WPV3

7A	GAYTCIATKGTIGAYTCBGT
6S	CCIAARCCRCAIAAYRGHC
P3W Probe-8A	**FAM**-CGYGTYTGYAYRGTRTCTGA-**BHQ1**

Appendix 3

Poliovirus rRT-PCR ITD 5.0 Kit

A kit to screen for wildtype and vaccine derived polioviruses in support of the Global Polio Eradication Initiative

Kit Components

The kit is supplied in one box containing six vials of primers and probes (Quadruplex EV+Sabin, PanPV, WPV1, PV Type 2, AFR WPV3, and SOAS WPV3). The box also contains five positive controls, tubes of water and one copy of this package insert. Positive controls: Sabin/PanPV positive control RNA, WPV1 positive control RNA, AFR WPV3 positive control RNA, SOAS WPV3 positive control RNA. The Sabin/PanPV control is used for the Quadruplex EV+Sabin, PanPV and the PV Type 2 assay (NEW!).
Additional required reagents and enzymes that are not supplied with the kit: Quanta Biosciences qScript XLT One-Step RT-qPCR ToughMix (CAT#95132-500) or Promega GoTaq® Probe 1-Step RT-qPCR System (CAT#A6120). The listed products were used in the development and evaluation of this kit and do not constitute a specific product endorsement. Kit availability from manufacturers may vary with each laboratory. It is the responsibility of each laboratory to find appropriate substitutes when necessary. Both kits were evaluated for performance on the ABI 7500 cycler.

Real-Time RT-PCR Reactions

1. Fill out PCR worksheet with name, date, primers, samples, and sample order, as well as thermocycler and program identifiers.
    a. Name wells using thermocycler software for samples and controls (positive and reagent).
    b. One positive control: non-infectious control RNA supplied with Polio rRT-PCR kit.
2. Thaw virus isolates and PCR reagents at room temperature. Keep all enzymes on ice while setting up.
3. Making enzyme mix: Label 1.5mL Eppendorf tubes for each primer assay. In a clean bio-safety cabinet,
    a. **Promega GoTaq® Probe 1-Step RT-qPCR System**: Mix 10µL of GoTaqProbe qPCR Master Mix, 1µL of primers/probe mix, 0.4µL of GoScript RT Mix and 7.6µL of nuclease free water. Dispense 19µL of reaction solution into each tube. Add 1 µl of template for a total volume of 20 µl.
    b. **Quanta ToughMix kit**: Mix 10µL of ToughMix, 1µL of primers/probe mix and 8µL of water. Dispense 19µl of reaction solution into each tube.
4. For large sample numbers: Create a master mix for each primer/probe set by multiplying the total number of specimens to determine the buffer volume. For example:
    a. **GoTaq® Probe 1-Step RT-qPCR System**: 10 samples x 10µL of GoTaqProbe qPCR Master Mix = 100µL, 4.0µL of GoScript RT Mix 1-Step RT-qPCR= 4µL, 1µL of primers/probe mix = 10µL and 7.6µL of dH_2O = 76µL. Dispense 19µL into each reaction tube.
    b. **ToughMix kit**: 10 samples x 10µL of Toughmix = 100µL, 1µL of primers/probe mix = 10µL and 8µL of dH_2O = 80µL. Dispense 19µL into each reaction tube.
5. Sample preparation: Take 40µl virus cell culture and place into a microtube and spin it (bench top micro centrifuge at 5,000 rpm or full speed (max rpm 6,400) of Tube-Strip PicoFuge) at room temperature for 2 minutes. (**Once the samples have been spun, they can be stored at -20°C and re-used if needed. You need to re-spin the sample after being stored at -20ºC).**
6. Take 1µl of cell culture supernatant (or 1µl of Control RNA) for each sample and add into the appropriate reaction strip/plate well. rRT-PCR does **NOT** require a 95°C heat step. One µl of extracted RNA can be used, but it's not generally required.
7. Place strips in real-time thermocycler and cycle as shown below. If using a thermocycler with a rapid ramp speed, program the ramp from 50°C to 72°C for 45 sec (note for the ABI 7500, you can use 25% ramp speed between the anneal and extension temperatures for all assays).
   Thermocyclers with regular ramp speeds can use the default ramp time; Stratagene Mx3000P and similar machines do not have adjustable ramp capabilities. An additional intermediate step between the lower and higher temperature in the PCR cycle compensates for the inability to adjust the ramp time between anneal and extension):
    a. RT reaction, 50°C, 30 minutes. (NEW!)
    b. Inactivate RT, 95°C, 1 minute. (NEW!)
    c. PCR cycles **(all primer sets)**:

   Using an ABI 7500:
   95°C for 15 seconds, 50°C for 45 seconds, and then a 25% ramp speed to 72°C for 5 seconds for **40 cycles**. **The end point fluorescent data is collected at the end of the 50°C anneal step**.

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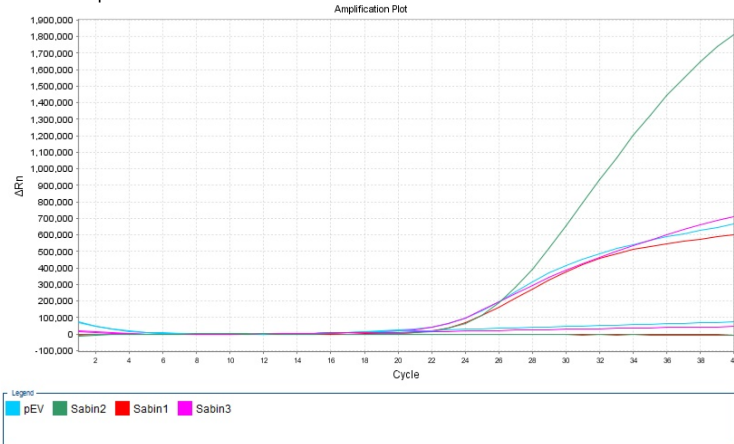
d. Select the appropriate dye filter to correspond with the assay being used. (Note: Quadruplex uses four channels, CY5, FAM, ROX, VIC; WPV1 only uses FAM; PV Type 2 uses FAM; AFR WPV3 uses CY5; SOAS WPV3 uses FAM).

e. Start run.

Interpretation

To validate your data first check the negative and positive control. The cycle threshold value (C_t) is the cycle number where the fluorescence rises above the background. These C_t values are calculated automatically by the Stratagene (or ABI 7500) software. However, you may have to manually adjust the baseline and C_t threshold to reflect your negative and positive controls (this is especially true with the ABI 7500 software). The C_t value cutoff is 32. You are looking for an S-shaped curve as shown in the examples below. Samples with C_t values from 28-32 should be re-analyzed using extracted RNA. Samples which have a C_t value <32 but have a flat fluorescence or an abnormal profile are most likely negative, and should be repeated using extracted RNA.

Below is an example with an ABI 7500.



Troubleshooting-Common Errors

Problem	Possible causes
All reactions negative, including positive control.	Component missing, wrong thermocycler profile used or bad reagent.
No C_t value(i.e. negative) with positive control; some sample reactions positive.	Control RNA degraded or not added.
Positive C_t values with one or more Sabin pairs, but PanPV is negative.	Ensure that RT-PCR was performed with 50°C annealing temperature. Ensure that ramp time for 50°C to 72°C step is approximately 40-45 seconds (~0.4ºC/sec).
Failure to select the correct dye filter for an assay.	The ABI 7500 will record all dyes regardless which dye is selected. Select the correct filter and re-analyze results.
No fluorescence data collected.	Bubbles in the well (or on the cap). Inhibition of rRT-PCR due to cell debris in the sample or too much clarified cell culture used.
Positive reaction (C_t value) with PanPV primers, but all other pairs are negative. Or if results are still discordant after repeating experiment one time	The isolate should be referred to a Specialized Reference Laboratory for identification.

Real-time PCR primer and probe sequences

Primers/Probes	Sequence (5'-3')
Quadruplex + EV	
Sabin 1 2S	AGG TCA GAT GCT TGA AAG C
Sabin 1 A4 **(CY5)**	CGC CCC CAC CGT TTC ACG GA
Sabin 1 1A	CCA CTG GCT TCA GTG TTT
Sabin 2 2S	CCG TTG AAG GGA TTA CTA AA
Sabin 2 (**FAM**)	ATT GGT TCC CCC GAC TTC CAC CAA T
Sabin 2 1A	CGG CTT TGT GTC AGG CA
Sabin 3 2S	AGG GCG CCC TAA CTT T
Sabin 3 **(ROX)**	TCA CTC CCG AAG CAA CAG
Sabin 3 1A	TTA GTA TCA GGT AAG CTA TC
PAN-EV S	GGC CCC TGA ATG CGG CTA ATC C
PAN-EV Probe **(VIC)**	CCG ACT ACT TTG GGW GTC CGT GT
PAN-EV A	GCG ATT GTC ACC ATW AGC AGY CA
Pan Poliovirus	
PanPV/PCR-S1	TTG GAG TTC TTC ACI TAI TCI MGI TTY GAY ATG
PanPV PCR-1A Probe **(FAM)**	TGR TTN ARI GCR TGI CCR TTR TT
PanPV PCR-1A	GGA GCT CCG GGT GGG AYR TAC ATI ATY TGR TAI AC
WPV1	
AFRO WPV1-S	GTA CAA ACC AGT CAY GTN AT
SOAS WPV1-S	CGT ACA GAC TAG RCA YGT NAT
WPV1-PROBE **(FAM)**	CAT WAT GGT TAC RCA MGC ACC T
WPV1-A	GAG AAT AAY TTG TCY TTK GAY GT
Poliovirus Type 2	
PV Type 2 S	GAT GCA AAY AAC GGI CAT GC
PV Type 2 PROBE S **(FAM)**	ATG ACT ATA CGT GGC AGA C
PV Type 2 PROBE S 1D **(FAM)**	CRC CKA TIC CTG GYA
PV Type 2 A	TCA TAA AAG TGG GAR TAC GCR TT
PV Type 2 A 1C	TCG TAA AAA TGA GAA TAT GCA TT
AFR WPV3	
SOAS WPV3-S	CAG GGA GTA GAT GAY CTN AT
AFRO WPV3-S	CAG GGG GTT GAT GAY TTR AT
WPV3-PROBE **(Cy5)**	CNC ARA ACA GYC TTC CGG ATA CC
WPV3-A	ACK GTG TCT GAY GGN AC
SOAS WPV3	
SOAS-6S	GTY RTA CAR CGR CGY AGY AGR A
SOAS-P3W 7S **(FAM)**	TTC TTY GCA AGI GGR GCR TGY GT
SOAS-5A	TCY TTR TAI GTR ATG CGC CAA G

PCR Positive Control RNA

Positive controls should be reconstituted before initial usage. Briefly spin the tubes to concentrate the lyophilized pellet before resuspension. Each lyophilized control should be reconstituted in 100µL dH_2O (provided in the kit). After addition of dH_2O, place in -20ºC overnight to allow proper rehydration of the RNA pellet. This solution should then be aliquoted into smaller volumes and stored at -20°C for future usage. Aliquoting will reduce risk of cross-contamination and the RNA hydrolyzing. The Sabin/PanPV positive control RNA (2 tubes) can be used for the Quadruplex EV+ Sabin assay, the PanPV assay and the PV Type 2 assay (NEW).

Contents of the rRT-PCR ITD 5.0 Kit

Tube Description	Volume	Cap Color	No. /kit (box)
Quadruplex EV + Sabin primers/probes	100µL	Yellow	1
Sabin/PanPV positive control RNA	*	Orange	2
PanPV primers/probe	100µL	White	1
WPV1 primers/probes	100µL	Blue	1
WPV1 positive control RNA	*	Light Blue	1
PV Type 2 primers/probe	100µL	Teal	1
AFR WPV3 primers/probe	100µL	Purple	1
AFR WPV3 positive control RNA	*	Light Purple	1
SOAS WPV3 primers/probe	100µL	Green	1
SOAS WPV3 positive control RNA	*	Light Green	1
Sterile, RNase free Water	1000µL	No Color Insert	7

*Lyophilized

References

1. Kilpatrick, D. R., K. Ching, J. Iber, R. Campagnoli, C.J. Freeman, N.Mishrik, H. Liu, , M. A. Pallansch, and O. M. Kew. 2004. Multiplex PCR Method for Identifying Recombinant Vaccine-related Polioviruses. J. Clin. Microbiol. 42:4313-4315.
2. Kilpatrick, D. R., C. F. Yang, K. Ching, A. Vincent, J. Iber, R. Campagnoli, M. Mandelbaum, L. De, A. Nix, and O. M. Kew. 2009. Rapid Group, Serotype and Vaccine Strain-Specific Identification of Poliovirus Isolates by Real-Time Reverse Transcription-PCR Using Degenerate Primers and Probes Containing Deoxyinosine Residues. J. Clin. Microbiol. 47(6): 1939-1941.
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7. Yang, C.-F., L. De, S.-J. Yang, J. Ruiz Gómez, J. Ramiro Cruz, B. P. Holloway, M. A. Pallansch, and O. M. Kew. 1992. Genotype-specific in vitro amplification of sequences of the wild type 3 polioviruses from Mexico and Guatemala. Virus Res. 24:277-296.
8. Expanded Programme on Immunization. 2000. Molecular characterization of polioviruses (laboratory manual). World Health Organization, Geneva

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Contact at CDC: Everardo Vega, email: EVega@cdc.gov; Steve Oberste, email: SOberste@cdc.gov.

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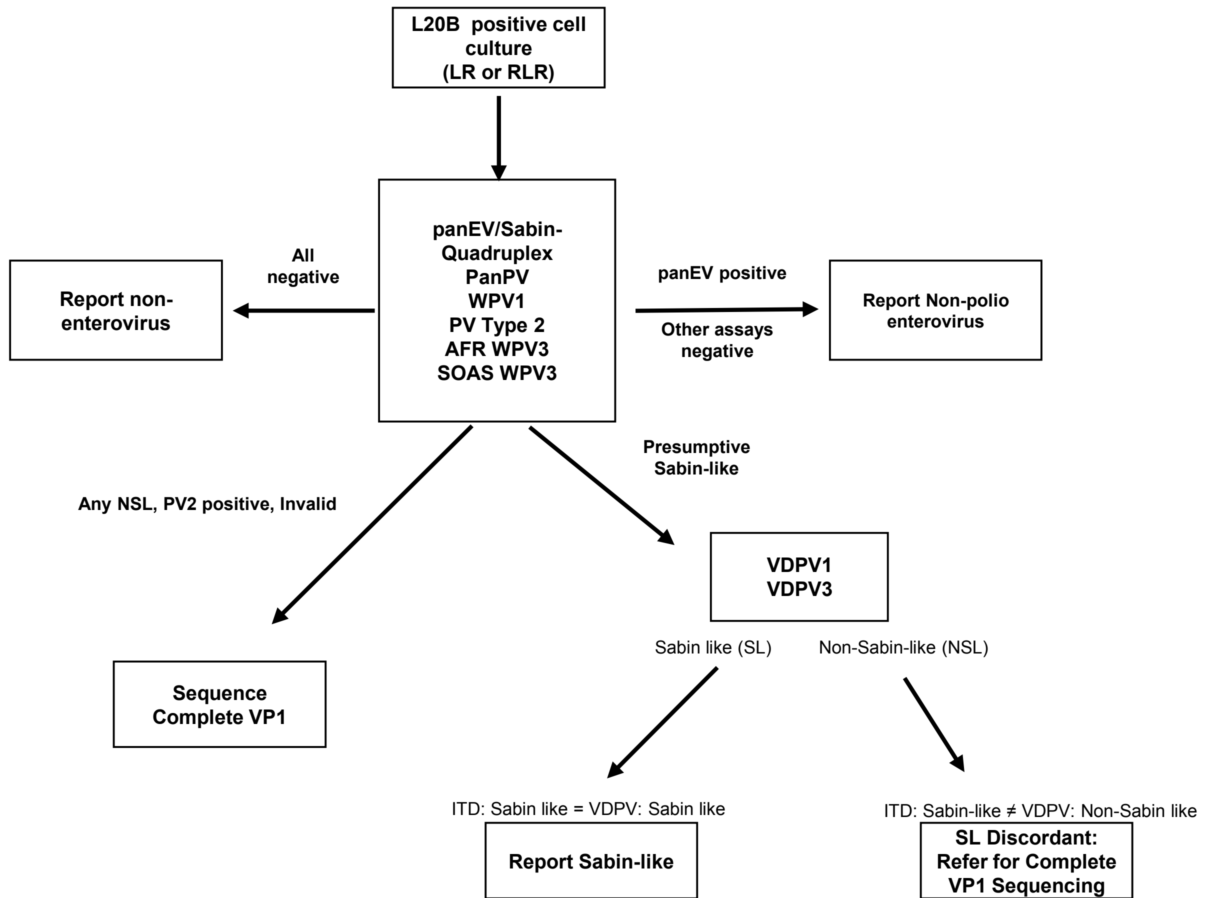
ITD 5.0 Algorithm



ITD 5.0 results*

PanEV	PanPV	Sabin1	Sabin2	Sabin3	WPV1	SOAS WPV3	AFR WPV3	PV Type 2	Report	Action
-	-	-	-	-	-	-	-	-	NEV	Report
+	-	-	-	-	-	-	-	-	NPEV	Report
+	+	-	-	-	-	-	-	-	Indeterminate	Refer for sequencing
+	+	+	-	-	-	-	-	-	SL1	VDPV test
+	+	-	+	-	-	-	-	+	PV2	Refer for sequencing
+	+	-	-	+	-	-	-	-	SL3	VDPV test
+	+	-	-	-	+	-	-	-	NSL1	Refer for sequencing
+	+	-	-	-	-	+	+	-	NSL3	Refer for sequencing
+	+	-	-	-	-	+	-	-	NSL3	Refer for sequencing
+	+	-	-	-	-	-	+	-	NSL3	Refer for sequencing
+	+	-	-	-	-	-	-	+	PV2	Refer for sequencing

*single results, does not include VDPV or final results after VDPV assay

Appendix 5

Poliovirus VDPV 5.0 rRT-PCR Kit

A kit to screen for vaccine-derived polioviruses
in support of the Global Polio Eradication Initiative

Kit Components

The kit is supplied in one box containing two vials of primers and probes (S1 VDPV and S3 VDPV). The box also contains two positive controls to correspond with the primer sets, tubes of water and one copy of this package insert.
Additional required reagents and enzymes that are not supplied with the kit: Quanta Biosciences qScript XLT One-Step RT-qPCR ToughMix (CAT#95132-500) or Promega GoTaq® Probe 1-Step RT-qPCR System (CAT#A6120). The listed products were used in the development and evaluation of this kit and do not constitute a specific product endorsement. Kit availability from manufacturers may vary with each laboratory. It is the responsibility of each laboratory to find appropriate substitutes when necessary. Both kits were evaluated for performance on the ABI 7500 cycler.

Real-Time RT-PCR Reactions

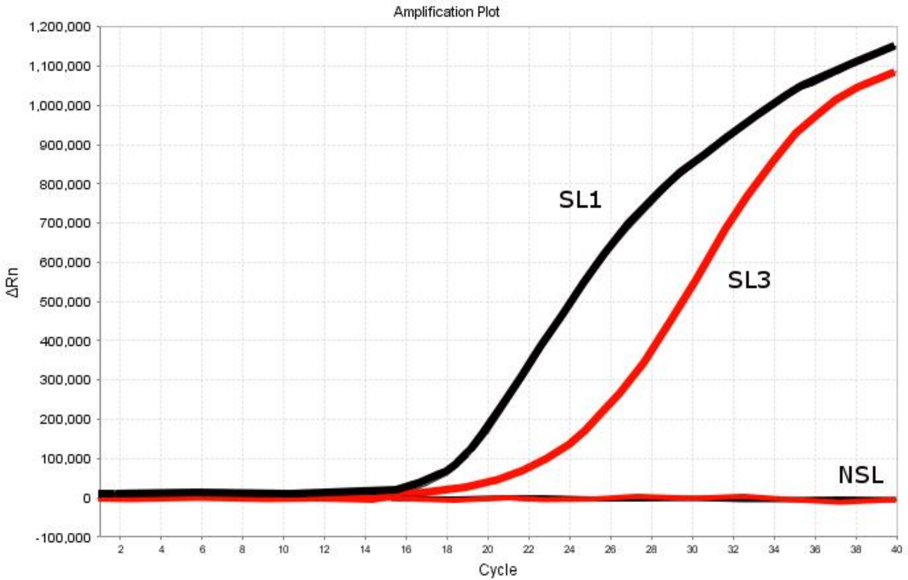
1. Fill out PCR worksheet with name, date, primers, samples, and sample order, as well as thermocycler and program identifiers.
    a. Name wells using thermocycler software for samples and controls (positive and reagent).
    b. One positive control: non-infectious control RNA supplied with Polio rRT-PCR kit.
2. Thaw virus isolates and PCR reagents at room temperature. Keep all enzymes on ice while setting up.
3. Making enzyme mix: Label 1.5mL Eppendorf tubes for each primer assay. In a clean bio-safety cabinet,
    a. **Promega GoTaq® Probe 1-Step RT-qPCR System**: Mix 10μL of GoTaqProbe qPCR Master Mix, 1μL of primers/probe mix, 0.4μL of GoScript RT Mix and 7.6μL of nuclease free water. Dispense 19μL of reaction solution into each tube. Add 1 μl of template for a total volume of 20 μl.
    b. **Quanta ToughMix kit**: Mix 10μL of ToughMix, 1μL of primers/probe mix and 8μL of water. Dispense 19μl of reaction solution into each tube.
4. For large sample numbers: Create a master mix for each primer/probe set by multiplying the total number of specimens to determine the buffer volume. For example:
    a. **GoTaq® Probe 1-Step RT-qPCR System**: 10 samples x 10μL of GoTaqProbe qPCR Master Mix = 100μL, 4.0μL of GoScript RT Mix 1-Step RT-qPCR= 4μL, 1μL of primers/probe mix = 10μL and 7.6μL of dH_2O = 76μL. Dispense 19μL into each reaction tube.
    b. **ToughMix kit**: 10 samples x 10μL of Toughmix = 100μL, 1μL of primers/probe mix = 10μL and 8μL of dH_2O = 80μL. Dispense 19μL into each reaction tube.
5. Sample preparation: Take 40μl virus cell culture and place into a microtube and spin it (bench top micro centrifuge at 5,000 rpm or full speed (max rpm 6,400) of Tube-Strip PicoFuge) at room temperature for 2 minutes. (**Once the samples have been spun, they can be stored at -20°C and re-used if needed. You need to re-spin the sample after being stored at -20ºC).**
6. Take 1μl of cell culture supernatant (or 1μl of Control RNA) for each sample and add into the appropriate reaction strip/plate well. rRT-PCR does NOT require a 95°C heat step. One μl of extracted RNA can be used, but it's not generally required.
7. Place strips in real-time thermocycler and cycle as shown below. If using a thermocycler with a rapid ramp speed, program the ramp from 50°C to 72°C for 45 sec (note for the ABI 7500, you can use 25% ramp speed between the anneal and extension temperatures for all assays).
   Thermocyclers with regular ramp speeds can use the default ramp time; Stratagene Mx3000P and similar machines do not have adjustable ramp capabilities. An additional intermediate step between the lower and higher temperature in the PCR cycle compensates for the inability to adjust the ramp time between anneal and extension):
    a. RT reaction, 50°C, 30 minutes. (NEW!)
    b. Inactivate RT, 95°C, 1 minute. (NEW!)
    c. PCR cycles **(all primer sets)**:

   Using an ABI 7500:
   95°C for 15 seconds, 50°C for 45 seconds, and then a 25% ramp speed to 72°C for 5 seconds for **40 cycles**. **The end point fluorescent data is collected at the end of the 50°C anneal step**.
    d. Select the FAM dye for each assay. Start run.

Interpretation

To validate your data first check the negative and positive control. The cycle threshold value (C_t) is the cycle number where the fluorescence rises above the background. These C_t values are calculated automatically by the Stratagene (or ABI 7500) software. However, you may have to manually adjust the baseline and C_t threshold to reflect your negative and positive controls (this is especially true with the ABI 7500 software). The C_t value cutoff is 32. You are looking for an S-shaped curve as shown in the examples below. Samples with C_t values from 28-32 should be re-analyzed using extracted RNA. Samples which have a C_t value <32 but have a flat fluorescence or an abnormal profile are most likely negative, and should be repeated using extracted RNA.

Below is an example with an ABI 7500.



Interpretation of VDPV Results

VP1	Result
Positive	SL (Report)
Negative	NSL (Report and Refer for Sequencing)

Troubleshooting-Common Errors

Problem	Possible causes
All reactions negative, including positive control	Component missing, wrong thermocycler profile used or bad reagent.
No C_t value(i.e. negative) with positive control; some sample reactions positive	Control RNA degraded or not added.
Failure to select the correct dye filter for an assay	The ABI 7500 will record all dyes regardless which dye is selected. Select the correct filter and re-analyze results
No fluorescence data collected	Bubbles in the well (or on the cap) Inhibition of rRT-PCR due to cell debris in the sample or too much clarified cell culture used
Results are still discordant after repeating experiment one time	The isolate should be referred to a Specialized Reference Laboratory for identification.

Sabin VDPV Real-time

Primer specificity	Primer & Probe sequences 5'-3'	
S1 VDPV VP1	Sense	CATGCGTGGCCATTATA
	Anti-Sense	TAAATTCCATATCAAATCTA
	22S VP1 Probe	**FAM**-CACCAAGAATAAGGATAAG -**BHQ1**
S3 VDPV VP1	Sense	CATTTACATGAAACCCAAAC
	Anti-Sense	TGGTCAAACCTTTCTCAGA
	12S VP1 Probe	**FAM**-AGGAACAACTTGGAC-**BHQ1**

PCR Positive Control RNA

Positive controls should be reconstituted before initial usage. Briefly spin the tubes to concentrate the lyophilized pellet before resuspension. Each lyophilized control should be reconstituted in 100µL dH_2O (provided in the kit). After addition of dH_2O, place in -20ºC overnight to allow proper rehydration of the RNA pellet. This solution should then be aliquoted into smaller volumes and stored at -20°C for future usage. Aliquoting will reduce risk of cross-contamination and the RNA hydrolyzing.

VDPV 4.0 rRT-PCR Kit

Tube Description	Vol.	Cap	# /box
S1 VDPV primers/probe	100µL	Yellow	1
PV1 positive control RNA	lyophilized	Light Purple	1
S3 VDPV primers/probe	100µL	Red	1
PV3 positive control RNA	lyophilized	Green	1
Sterile, RNase free Water	1000µL	No Color Insert	2

References

1. Kilpatrick, D. R., K. Ching, J. Iber, R. Campagnoli, C.J. Freeman, N.Mishrik, H. Liu, , M. A. Pallansch, and O. M. Kew. 2004. Multiplex PCR Method for Identifying Recombinant Vaccine-related Polioviruses. J. Clin. Microbiol. 42:4313-4315.
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Supplemental ITD PV Type 2 rRT-PCR Kit

A kit to screen for Type 2 polioviruses in support of the Global Polio Eradication Initiative

Kit Components

The kit is supplied in one box containing one vial of primers and probes (PV Type 2). The box also contains one positive control to correspond with the primer set, one tube of water and one copy of this package insert.

Additional required reagents and enzymes that are not supplied with the kit: Quanta Biosciences qScript XLT One-Step RT-qPCR ToughMix (CAT#95132-500) or Promega GoTaq® Probe 1-Step RT-qPCR System (CAT#A6120). The listed products were used in the development and evaluation of this kit and do not constitute a specific product endorsement. Kit availability from manufacturers may vary with each laboratory. It is the responsibility of each laboratory to find appropriate substitutes when necessary. Both kits were evaluated for performance on the ABI 7500 cycler.

Real-Time RT-PCR Reactions

1. Fill out PCR worksheet with name, date, primers, samples, and sample order, as well as thermocycler and program identifiers.
   a. Name wells using thermocycler software for samples and controls (positive and reagent).
   b. One positive control: non-infectious control RNA supplied with Polio rRT-PCR kit.
2. Thaw virus isolates and PCR reagents at room temperature. Keep all enzymes on ice while setting up.
3. Making enzyme mix: Label 1.5mL Eppendorf tubes for each primer assay. In a clean bio-safety cabinet,
   a. **Promega GoTaq® Probe 1-Step RT-qPCR System**: Mix 10µL of GoTaqProbe qPCR Master Mix, 1µL of primers/probe mix, 0.4µL of GoScript RT Mix and 7.6µL of nuclease free water. Dispense 19µL of reaction solution into each tube. Add 1 µl of template for a total volume of 20 µl.
   b. **Quanta ToughMix kit**: Mix 10µL of ToughMix, 1µL of primers/probe mix and 8µL of water. Dispense 19µl of reaction solution into each tube.
4. For large sample numbers: Create a master mix for each primer/probe set by multiplying the total number of specimens to determine the buffer volume. For example:
   a. **GoTaq® Probe 1-Step RT-qPCR System**: 10 samples x 10µL of GoTaqProbe qPCR Master Mix = 100µL, 4.0µL of GoScript RT Mix 1-Step RT-qPCR= 4µL, 1µL of primers/probe mix = 10µL and 7.6µL of dH_2O = 76µL. Dispense 19µL into each reaction tube.
   b. **ToughMix kit**: 10 samples x 10µL of Toughmix = 100µL, 1µL of primers/probe mix = 10µL and 8µL of dH_2O = 80µL. Dispense 19µL into each reaction tube.
5. Sample preparation: Take 40µl virus cell culture and place into a microtube and spin it (bench top micro centrifuge at 5,000 rpm or full speed (max rpm 6,400) of Tube-Strip PicoFuge) at room temperature for 2 minutes. (**Once the samples have been spun, they can be stored at -20°C and re-used if needed. You need to re-spin the sample after being stored at -20ºC).**
6. Take 1µl of cell culture supernatant (or 1µl of Control RNA) for each sample and add into the appropriate reaction strip/plate well. rRT-PCR does **NOT** require a 95°C heat step. One µl of extracted RNA can be used, but it's not generally required.
7. Place strips in real-time thermocycler and cycle as shown below. If using a thermocycler with a rapid ramp speed, program the ramp from 50°C to 72°C for 45 sec (note for the ABI 7500, you can use 25% ramp speed between the anneal and extension temperatures for all assays).
   Thermocyclers with regular ramp speeds can use the default ramp time; Stratagene Mx3000P and similar machines do not have adjustable ramp capabilities. An additional intermediate step between the lower and higher temperature in the PCR cycle compensates for the inability to adjust the ramp time between anneal and extension):
   a. RT reaction, 50°C, 30 minutes.
   b. Inactivate RT, 95°C, 1 minute.
   c. PCR cycles:
   Using an ABI 7500:
   95°C for 15 seconds, 50°C for 45 seconds, and then a 25% ramp speed to 72°C for 5 seconds for **40 cycles**. **The end point fluorescent data is collected at the end of the 50°C anneal step**.
   d. Select the FAM dye for the assay. Start run.

Interpretation

To validate your data first check the negative and positive control. The cycle threshold value (C_t) is the cycle number where the fluorescence rises above the background. These C_t values are calculated automatically by the Stratagene (or ABI 7500) software. However, you may have to manually adjust the baseline and C_t threshold to reflect your negative and positive controls (this is especially true with the ABI 7500 software). The C_t value cutoff is 32. You are looking for an S-shaped curve as shown in the examples below. Samples with C_t values from 28-32 should be re-analyzed using extracted RNA. Samples which have a C_t value <32 but have a flat fluorescence or an abnormal profile are most likely negative, and should be repeated using extracted RNA.

Interpretation of PV Type 2 Results

PV Type 2	Result
Positive	Report and Refer for Sequencing
Negative	Negative for Poliovirus Type 2

Troubleshooting-Common Errors

Problem	Possible causes
All reactions negative, including positive control	Component missing, wrong thermocycler profile used or bad reagent.
No C_t value(i.e. negative) with positive control; some sample reactions positive	Control RNA degraded or not added.
Failure to select the correct dye filter for an assay	The ABI 7500 will record all dyes regardless which dye is selected. Select the correct filter and re-analyze results
No fluorescence data collected	Bubbles in the well (or on the cap) Inhibition of rRT-PCR due to cell debris in the sample or too much clarified cell culture used

PV Type 2 Real-time Primers and Probes

Primer specificity	Primer & Probe sequences 5'-3'
PV Type 2 S	GAT GCA AAY AAC GGI CAT GC
PV Type 2 PROBE S **(FAM)**	ATG ACT ATA CGT GGC AGA C
PV Type 2 PROBE S 1D **(FAM)**	CRC CKA TIC CTG GYA
PV Type 2 A	TCA TAA AAG TGG GAR TAC GCR TT
PV Type 2 A 1C	TCG TAA AAA TGA GAA TAT GCA TT

PCR Positive Control RNA

The positive control should be reconstituted before initial usage. Briefly spin the tubes to concentrate the lyophilized pellet before resuspension. Each lyophilized control should be reconstituted in 100µL water (provided in the kit). Place in -20ºC overnight to allow proper rehydration of the RNA pellet. This solution should then be aliquoted into smaller volumes and stored at -20°C for future usage. Aliquoting will reduce risk of cross-contamination and the RNA hydrolyzing.

Supplemental ITD PV Type 2 rRT-PCR Kit

Tube Description	Volume	Cap	# /box
PV Type 2 primers/probes	100 µL	Orange	1
PV Type 2 Pos. Control		Black	1
RNase Free Water	1000 µL	No Color Insert	1

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