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Measurement of BK-polyomavirus non-coding control region driven transcriptional activity via flow cytometry

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Dear Dr. Bajaj,

Thank you for the opportunity to send our revised manuscript. Following this letter, please find the specific comments with our responses indicted in red. Changes made in the manuscript were marked using the Track Changes mode.

We now revised the result section of the video also included the title card at the end of the after the conclusion section.

We would be glad if you now consider our revised manuscript for publication in JoVE.

Sincerely,

Marek Widera, Ph.D.

TITLE:

Measurement of BK-polyomavirus Non-Coding Control Region Driven Transcriptional Activity via Flow Cytometry

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KEYWORDS:

BK-Polyomavirus, BKPyV, Non-coding control region, NCCR, bidirectional promoter activity, FACS, antiviral activity, renal transplantation, large T antigen, SV40

SUMMARY:

In this manuscript, a protocol is presented to perform FACS-based measurement of BK-polyomavirus transcriptional activity by using HEK293T cells transfected with a bidirectional reporter plasmid expressing tdTomato and eGFP. This method further allows to quantitatively determine the influence of novel compounds on viral transcription.

ABSTRACT:

Polyomaviruses, like the BK-polyomavirus (BKPyV), can cause severe pathologies in immunocompromised patients. However, since highly effective antivirals are currently not

available, methods measuring the impact of potential antiviral agents are required. Here, a dual fluorescence reporter that allows the analysis of the BKPyV non-coding control-region (NCCR) driven early and late promoter activity was constructed to quantify the impact of potential antiviral drugs on viral gene expression via tdTomato and eGFP expression. In addition, by cloning BKPyV-NCCR amplicons which in this protocol have been exemplarily obtained from the blood-derived DNA of immunocompromised renal transplanted patients, the impact of NCCR-rearrangements on viral gene expression can be determined. Following cloning of the patient derived amplicons, HEK293T cells were transfected with the reporter-plasmids, and treated with potential antiviral agents. Subsequently, cells were subjected to FACS-analysis for measuring mean fluorescence intensities 72 h post transfection. To also test the analysis of drugs that have a potential cell cycle inhibiting effect, only transfected and thus fluorescent cells are used. Since this assay is performed in large T Antigen expressing cells, the impact of early and late expression can be analyzed in a mutually independent manner.

INTRODUCTION:

Polyomaviruses represent an independent family of small double-stranded DNA (dsDNA) viruses with the Simian virus 40 (SV40) as a prototype species. The primary infection mainly occurs during the childhood, which usually proceed without disease symptoms and usually cause latent infections in immune competent hosts. The BK-polyomavirus (BKPyV) mainly persists in renal tubules cells without causing nephropathologies, however, in case of impairment of the immune-competence after renal transplantation the virus can reactivate and cause severe damages and impaired graft function when reaching a high viremia (1 x 10^4 BKPyV DNA copies/mL)[1,2]. In approximately 10% of kidney transplant recipients, reactivation of the BK-polyomavirus (BKPyV) results in a polyomavirus associated nephropathy (PyVAN), which was up to 80% associated with a high risk of renal allograft failures[3,4]. Since no approved antiviral agents are available, current therapy is based on the reduction of immunosuppression. Interestingly, mTOR inhibitors seem to have an antiviral effect; thus, switching the immunosuppressive therapy to mTOR-based immunosuppression might represent an alternative approach to prevent progression of the BKPyV viremia[5-7]. However, the mTOR-based antiviral mechanism is currently still incompletely understood. Thus, methods measuring the impact of potential antiviral agents in clinically relevant concentrations are required.

The circular genome of the BKPyV consists of approximately 5 kb harboring a non-coding control region (NCCR) that serves as an origin of replication and concomitantly a bidirectional promoter driving the expression of early and late phase mRNA transcripts. Since spontaneously occurring NCCR-rearrangements, deletions, and duplications are found in pathogenic BKPyV[8] and significantly accumulated in patients suffering from PVAN[5,9], a comparison of archetypical (wt) and re-arranged (rr) NCCR-activities are helpful to characterize viral replicative fitness.

As summarized in **Figure 1**, this protocol describes a commonly used method to measure BKPyV NCCR transcriptional activity by quantifying the fluorescence of two fluorophores tdTomato and eGFP expressed from a reporter plasmid[5,9-11]. The procedure is performed in the presence of the SV40 large T antigen (lTAg), which allows to analyze the impact of potential antiviral agents on the early and late NCCR-activity separately[5]. This assay further analyzes the impact of

rearrangements on the NCCR activity and comparison with wt-NCCRs[5,9]. The reporter plasmid harbors the SV40 late polyadenylation signal downstream of each fluorophore open reading frame to ensure comparable and efficient processing of both transcripts for tdTomato and eGFP, respectively. Compared to qRT-PCR based methods[5,12], this FACS-based approach represents a low cost and high throughput compatible alternative since no complicated extraction protocols for infected cell culture and no expensive antibodies for immune fluorescence staining are needed. Furthermore, since a defined amount of fluorescent cells are analyzed via flow cytometry, the analysis of cell cycle inhibiting agents is also possible in a quantitative manner.

PROTOCOL:

This protocol follows the guidelines of human research as approved by the ethic committee of the medical faculty of the University of Duisburg-Essen (14-6028-BO).

1. Collection of blood or urine samples and isolation of polyomavirus DNA

1.1. Collect at least 3 mL of blood in EDTA tubes or urine in acquisition tubes.

1.2. Centrifuge the sample at 2,500 *x g* for 15 min. If needed, pipette plasma into a new tube and store the plasma samples at 4 °C for several days or freeze at -20 °C for longer storage.

1.3. Prepare 40 µL of proteinase K into a 1.5 mL microcentrifuge tube and add 400 µL of plasma by pipetting.

1.4. Lyse the sample by adding 400 µL of lysis buffer, vortex for 15 s, and incubate for 10 min at 56 °C.

1.5. Isolate the DNA using a DNA blood extraction kit as described in the manufacturer's instructions. Briefly, add alcohol and load the lysates onto a spin column containing a DNA binding silica-based membrane. Wash the columns several times to yield pure DNA.

1.6. Elute the yielded DNA in 30-50 µL of TE buffer.

2. Amplification of the non-coding control region (NCCR)

2.1 Perform the following procedure in physically separated rooms. Use an isolated room to prepare the reagents and distribute the mix to the PCR tubes.

2.2 Prepare the Master Mix for the pre-PCR using primer pair A (**Table 1**) in a total volume of 50 µL and use the previously isolated DNA from step 1.6. The pipetting scheme for preparing the master mix for the pre and nested PCR is printed in **Table 2**.

2.3 Distribute 45 µL of the master mix into the PCR tubes.

2.4 Add 5 µL of the isolated DNA into the PCR tubes.

NOTE: Use another room than the one for the master mix preparation to avoid contamination.

2.5 Run the PCR with the reaction conditions as illustrated in **Table 3**. Repeat denaturation, annealing, and extension in 35 cycles.

2.6 For the nested amplification use primer pair B harboring the restriction sites for AgeI and SpeI (**Table 1**).

2.7 Run the nested PCR with the reaction conditions as described in steps 2.2 to 2.5 using 5 µL of the pre-PCR.

2.8 Mix 10 µL of the PCR product with 2 µL of 6x gel loading dye. Load 10 µL of the mix on a 1.5% agarose gel, and run the gel for 30 min at 60 mA.

2.9 Visualize the gel using an appropriate UV documentation system.

NOTE: The size of the amplicon is expected to be between 300-500 bp depending on whether rearrangements (deletions, insertions, or duplications) occurred (**Figure 2C**).

2.10 Purify the PCR amplicons using a PCR purification kit according to the manufacturer's instructions. Elute the PCR amplicons in 30 µL of elution buffer.

2.11 Send the amplicons for Sanger sequencing using primer pair B.

3. Cloning of the NCCR into the dual fluorescence reporter

3.1 Digest the purified amplicons (step 2.10) with AgeI and SpeI for 2 h at 37 °C as described in **Table 4**.

3.2 Repeat step 2.10 and purify the digested amplicons.

3.3 In parallel, also digest the plasmid backbone (1.5 µg) with AgeI and SpeI for 2 h at 37 °C as described in **Table 5**. This step only needs to be performed once. For subsequent reactions, freeze the digestion at -20 °C.

3.4 Analyze the digested plasmid backbone on a 0.8% low melt agarose gel.

NOTE: Do not run the gel with a current higher than 40 mA. The expected insert of the spacer region originally derived from the plasmid pEX-K4 2-LTR CD3[13] is 128 bp (**Figure 2C**).

3.5 Visualize DNA fragments using long-wave UV light (320 nm) and cut out the backbone band using a clean scalpel. Transfer the low melt gel fragment into a new 1.5 mL microcentrifuge tube.

Avoid long UV exposure times to prevent DNA damage.

NOTE: Since low melt agarose is used, it is not necessary to purify the DNA before ligation.

3.6 Heat the backbone containing the low melt agarose piece for 10 min at 65 °C in order to melt the gel piece and mix every 2 min by gentle vortexing. The melted gel can be directly used for ligation.

3.7 Ligate the backbone and the digested amplicon using T4 DNA ligase over night at 16 °C using the scheme shown in **Table 6**.

3.8 Perform transformation in *E. coli* using the heat shock method, plate bacteria on LB-amp plates, and culture at 37 °C over night.

3.9 On the next day, select three positive clones and prepare overnight *E. coli* cultures (5 mL of LB-Amp) and culture at 37 °C.

3.10 Isolate the plasmid-DNA using standard protocols as described elsewhere[14], perform AgeI and SpeI digestion to check for positive clones and visualize cut out fragments on a 1% agarose gel. The spacer band (128 bp) will be replaced by the larger NCCR-sequence (300-500 bp, see above).

3.11 Send the plasmid for Sanger sequencing using primer EGFP-N or primer pair B (**Table 1**).

3.12 Since mutations might spontaneously occur, compare the sequencing results with the sequencing results obtained with the amplicon. Only use the clones containing identical sequences compared to the amplicon.

3.13 Use a molecular workbench software (e.g., freeware GENtle 1.9.4 or other sequence editing programs) to align and edit the obtained sequences (**Figure 3**).

3.14 Start GENtle 1.9.4 and import the DNA sequences by clicking on the **Import** button (green down arrow).

3.15 Next click on **Tool** and select **Alignment** from the menu (Use Ctrl+G as a shortcut) and choose the DNA sequences for alignment.

3.16 Add a sequence to the alignment by clicking on **Add** or remove a sequence by clicking on **Remove**. Include an archetypical NCCR consensus sequence like *JN192438*[15], do not use the NCCR-sequence from the commonly used Dunlop-strain[16], since it harbors rearrangements and duplications others than the archetypical BKPyV strains.

NOTE: The NCCR already contains the translational start codon (**Figure 3**).

3.17 Choose a method for the alignment by clicking on **Algorithm** in the toolbar and choose clustal W. Set the alignment parameters to **match 2; gap extension penalty -1; gap penalty -2** and click on **OK** to run the alignment.

4. Transient transfection of HEK293T cells with the reporter plasmid and treatment with potential antiviral agents

4.1 To prepare sufficient amounts of plasmid DNA for subsequent transfection experiments prepare a 150 mL overnight culture. Isolate the plasmid DNA using a plasmid isolation kit. Alternatively, other plasmid purification kits may be used.

4.2 Seed 1 x 10^5 HEK293T cells in DMEM containing 10% FCS, penicillin and streptomycin (1x) per well of a 12-well plate 24 h prior to transfection and incubate overnight at 37 °C and 5% CO_2 to maintain active proliferation during transfection.

NOTE: Cells should be approximately 80% confluent at transfection.

4.3 Place 250 µL of reduced serum media in a sterile tube and add 1 µg of each reporter plasmid DNA and mix gently by pipetting.

4.4 Add 3 µL of the transfection reagent to the DNA mixture, mix gently by pipetting and incubate for 15 min. Pre warm the transfection reagent to the ambient temperature of 22 °C and vortex gently before use.

4.5 Add the mixture drop-wise to the wells and gently distribute to the well.

4.6 After 4 h, replace the supernatant with fresh medium containing the testing agents and solvent control. Incubate at 37 °C until analysis. In this example, the mTOR inhibitors INK128 (100 ng/mL) and rapamycin (100 ng/mL) were used.

5. Fluorescence microscopy and flow cytometry

5.1 Check cells for the red and green fluorescence under the fluorescence microscope (**Figure 4**).

NOTE: Red and green fluorescence correspond to the early and late BKPyV gene expression, respectively.

5.2 After 72 h post transfection, aspirate the supernatant and wash the cells twice with 1 mL of cold PBS.

5.3 Gently add 500 µL of trypsin and turn the plate slightly to avoid premature detachment of the cells. This step is important to avoid cell doublets.

5.4 After addition, remove the trypsin directly with the same pipette tip.

5.5 Incubate the cells for at least 5 min at 37 °C.

5.6 Resuspend trypsinized cells with 1 mL of PBS containing 3% FCS and transfer the suspension in pre-labeled FACS tubes.

5.7 Add DAPI (1 µg/mL) prior to the FACS analysis.

5.8 Analyze the cells using a flow cytometer. For each sample, measure at least 10,000 living cells, which are negative for DAPI staining.

6. Data analysis

6.1 Import the data to the flow cytometry analysis software and add samples by **Click and Drag** into the workspace.

6.2 Double click on the imported file and create a graph plot. Choose FSC-A versus SSC-A by clicking on the x- and y-axis. Gate the main cell population by clicking on **Polygon** at the toolbar and framing the cell population (**Figure 5**). Name the chosen cell population as required (for example “single cells”). The “single cells” will appear as a new workspace.

6.3 Proceed with gating “single cells” for DAPI negative (i.e., “living cells”). To identify living cells compare the cell population with and without DAPI staining. In both plots choose FSC-A versus pacific-blue-A.

6.4 Click on the rectangle and choose the DAPI negative cell population, name the chosen cell population “living cells”, and create a new dot-plot showing FITC (eGFP) versus PE (tdTomato) as described before.

6.5 Add quadrants in “living cells” by choosing **Quad** from the toolbar. Gate the population by dragging the center of the quadrant to the edge of each population. The quadrants represent 1) eGFP-tdTOM-, 2) eGFP-tdTOM+, 3) eGFP+tdTOM-, 4) eGFP+tdTOM+.

NOTE: Due to the spectral overlay of emission spectra between FITC and PE, initial compensation is essential to distinguish between red and green signals and to achieve accurate results.

6.6 Determine mean fluorescence intensities (MFIs) by right clicking on the quadrant and choosing **Add Statistics**. Choose **Mean** and click **OK**. The mean MFI is now displayed below the population in the section “statistics”. Quadrants 2 and 4 correspond to early expression, while quadrants 3 and 4 represent late expression.

6.7 Add additional replicates in the same manner for statistical power.

6.8 For data interpretation plot MFI values of early and late into a bar plot:

6.9 To compare NCCR activities obtained from different donors or virus strains, add an archetypical control or the Dunlop strain[16] and set its relative MFIs to 100%, and calculate the relative MFI values. Repeat with each replicate and determine mean and standard deviation.

6.10 To evaluate an effect of potential compounds on the transcriptional activity, set the solvent-control to 100% and plot the MFI of the treated cells.

REPRESENTATIVE RESULTS:

In this representative experiment, the BK-polyomavirus Non-Coding Control Region driven transcriptional activity was measured via flow cytometry. In addition, a mTOR inhibitor, which might be used to treat patients after BKPyV reactivation, was tested for its inhibition of the viral early gene expression. To this end, a dual fluorescence-reporter assay was used as published previously[5]. The overall workflow scheme of the experimental setup is illustrated in **Figure 1**. First, blood samples from immunocompromised renal transplanted patients were collected according to the guidelines of human research as approved by the institutional ethics committee. The blood was collected in EDTA-containing tubes and the phases were separated by centrifugation. Subsequently, 400 µL of plasma was used for isolation of the polyomavirus DNA. The non-coding control region (NCCR) was amplified using a nested-PCR protocol as illustrated in **Figure 2A** using the outer primer pair BKV-CR-1fw and BKV-CR-1rv, as well as, the inner primer pair BKV-CR-2fw-AgeI (Primer AgeI) and BKV-CR-2rv-SpeI (Primer SpeI)[17], the latter of which harbors the restriction sites for AgeI and SpeI. The amplicon verification was performed via agarose gel electrophoresis as shown in **Figure 2B**. While the amplicons derived from archetypical NCCR sequences (wt) had a homogeneous size distribution in the gel, those derived from rearranged NCCRs with insertions (rr_{ins}) and deletions (rr_{del}) differed in their size (approx. 300-500 bp). Notably, due to rearrangements, the Dunlop NCCR reference sequence[16] (DUN), which was included as a control, was larger than the NCCR obtained from archetypical viruses. Since the amplicons corresponded to the predicted sizes, the purified PCR products were sent for Sanger sequencing for later comparison with the sequences cloned into the reporter plasmid for further analysis.

For cloning of the amplicons, digestion was performed using the two restriction enzymes AgeI and SpeI. After cloning into the dual fluorescence reporter, which was performed using standard cloning procedure, selection of positive clones containing the NCCR was verified by AgeI and SpeI digestion (**Figure 2C**). Here, the small spacer region (NC, 128 bp) was replaced by the larger NCCR-fragments (approx. 300-500 bp) indicating positive clones. Plasmid DNA isolated from verified clones were sent for Sanger sequencing and compared with the sequences obtained from amplicon sequencing (not shown). Only the clones that match the amplicon sequence were chosen for further processing. As depicted in **Figure 3,** the sequencing results were compared with an archetypical NCCR sequence (JN192438). In this example, a deletion in the P-block and a substitution in O-block were detected.

To subsequently test the transcriptional activity of the cloned NCCR sequences in cell culture, HEK293T cells were transiently transfected with the respective reporter constructs (**Figure 4A**).

As visualized in **Figure 4B**, the transfection efficiency and NCCR-activity were monitored via fluorescence microscopy. Red and green fluorescence corresponded to early and late BKPyV gene expression, respectively[5]. The cells expressed both fluorophores indicating efficient early and late promotor activity. As illustrated by the two examples, the first NCCR (NCCR1) displayed a strong late gene expression, while the second example (NCCR2) had a comparatively strong early but moderate late gene expression (**Figure 4B**). Thus, samples were prepared for the flow cytometry measurement. As described in protocol section 6, DAPI negative cells were gated (**Figure 5A**, lower panel) and a dot plot was created showing eGFP versus tdTomato. Samples that were negative (**Figure 5B**), as well as those positive for tdTomato (**Figure 5C**) or eGFP (**Figure 5D**) only, were included into the analysis. Note, initial compensation was performed, which is essential to distinguish red and green signals and to achieve accurate results[18]. Mean fluorescence intensities were derived from each testing clone. Here, tdTomato positive cells corresponded to early expression while eGFP positive cells represented late expression. In this example the treatment with the dual mTOR inhibitor INK128 significantly reduced tdTomato MFI (**Figure 5E-F**), which means that early expression was inhibited.

FIGURE AND TABLE LEGENDS:

Figure 1: Workflow scheme for the experimental setup. 1) Collection of blood (alternatively urine) samples, **2)** Isolation of polyomavirus DNA **3)** Amplification of the non-coding control region (NCCR) using a nested-PCR protocol, **4)** Agarose gel electrophoresis and amplicon verification, **5)** Sanger sequencing of the PCR amplicon, **6)** Cloning of the NCCR into the dual fluorescence reporter using AgeI and SpeI, **7)** Selection of positive clones, **8)** Sanger sequencing of the plasmid DNA, **9)** Transient transfection of HEK293T cells with the reporter plasmid and addition of compounds to be tested, **10)** Fluorescence microscopy to verify efficient transfection, **11)** Flow cytometry analysis, **12)** Gating and analysis of eGFP tdTomato expression, **13)** Data interpretation.

Figure 2: Nested PCR and representative analysis of patient derived NCCR-amplicons. A) DNA derived from immunocompromised renal transplanted patients was subjected to semi-nested PCR amplification using oligonucleotide primers linked with restriction sites AgeI and SpeI. Primer names and length (base pairs) of archetypical O, P, Q, R, and S-blocks from BKPyV strain JN192438 are indicated in brackets. **B)** Amplicons were analyzed by electrophoresis in 1.5% agarose gel and visualized using DNA staining. M_1 = 100 bp ladder, wt = wildtype (archetypical), rr = rearranged, ins = insertions, del= deletions, DUN = Dunlop Strain. **C)** Plasmid digestion with AgeI and SpeI. Digested fragments were analyzed by electrophoresis in 1.5% agarose gel and visualized using DNA staining. M_1 = 100 bp ladder, M_2 = 2-log ladder. NC = negative control (spacer). + = positive clone.

Figure 3: Cloning and validation of the NCCR into the dual fluorescence reporter. Representative results of plasmid sequencing. Amplicons were purified using a PCR purification kit and subjected to Sanger sequencing. The respective sequences were aligned to the NCCR sequence derived from archetypical BKPyV strain JN192438. Deletions and substitutions are marked in red. The AgeI and SpeI restriction sites, translational start of eGFP open reading frame (printed in bold) as

well as the NCCR blocks O-S are indicated. The respective blocks and the restriction sites are highlighted in color. The length of each NCCR block in base pairs are printed in brackets.

Figure 4: Representative fluorescence microscopy analysis of early and late NCCR promoter activity. A) Gene map of the dual fluorescence reporter under the control of the bidirectional promoter. As described previously[5] the plasmid pTA-tdTOM-NCCR-eGFP (6,009 bp) harbors the SV40 late polyadenylation signal downstream of each expression cassette to ensure comparable and efficient processing of both transcripts for tdTomato (early expression) and eGFP (late expression), respectively. NCCR is flanked by AgeI and SpeI. The vector contains an ampicillin resistance cassette for selection during cloning procedure. **B)** HEK293T cells were transfected with dual-fluorescence reporter constructs each under the control of patient derived NCCR sequences (NCCR1-2). Cells were subjected to brightfield and fluorescence microscopy analysis 72 h post transfection. Filter settings of the microscope were used in the following excitation ranges: green for tdTomato (515-560 nm) and blue for eGFP (450-490 nm). The scale bar (200 µm) is indicated in the bottom right of each photography.

Figure 5: Representative FACS analysis. Shown is the simple gating strategy required for this method. Two-parameter density or dot plots are used. **A)** First, FSC is plotted against Pacific Blue, while only DAPI negative (i.e., living cells) are further processed. **B-F)** FITC (eGFP) versus PE (tdTomato) are plotted. **C-D)** Add exclusively green and red fluorescent cells to adjust the compensation on the flow cytometer. **E-F)** In this example, the mTOR inhibitors INK128 and significantly reduces the tdTomato MFI, which corresponds to a reduction of the BKPyV early gene expression.

Table 1: Oligonucleotides used in this protocol. The underlined bases indicate the restriction enzyme sites for AgeI and SpeI, respectively.

Table 2: Master mix preparation protocol. The instruction applies to both pre- and nested PCR reactions as described in steps 2.2 and 2.7, respectively.

Table 3: PCR program used for NCCR amplification. The instruction applies to both pre- and nested PCR reactions.

Table 4: Amplicon digestion protocol. The instruction applies for the simultaneous digestion of the amplicon DNA with two restriction enzymes described in step 3.1.

Table 5: Plasmid digestion protocol. The instruction applies for the simultaneous digestion of the plasmid DNA backbone with two restriction enzymes described in step 3.3.

Table 6: Plasmid ligation protocol. The instruction applies for the ligation of the plasmid DNA backbone with the digested PCR amplicon DNA described in step 3.7.

DISCUSSION:

In this article, a commonly used method is presented that allows for the analysis of the BKPyV

non-coding control-region (NCCR) driven early and late promoter activity. The NCCR activity can be measured simultaneously and does not need lysis of the transfected cells. Furthermore, a relatively large number of cells can be analyzed and the co-transfection of additional markers for normalization of the fluorescence values is not necessary.

A critical part of this method is that the cloned NCCR should contain exactly the same sequences as identified by the amplicon sequencing, which corresponds to the majority variants present in the patient plasma. To achieve accurate results and to minimize PCR-prone errors it is highly recommended to use a polymerase with proof reading activity. Alternatively, sequences can be ordered by commercial gene synthesis services. The designated NCCR sequences can be ordered including flanking AgeI and SpeI restriction sites for direct cloning. In this case, digest the plasmid parallel to the backbone plasmid and replace the spacer insert with the corresponding NCCR fragment from synthesized construct. DNA can be alternatively isolated from urine samples. However, since BKPyV is also secreted into urine by immunocompetent individuals and not necessarily reflect active replication, the clinical relevance is limited. Ideally, the DNA can also be isolated from renal biopsies in which previously PVAN could be histologically detected (compare with Korth et al., 2019[19]).

By measuring the mean fluorescence intensity of green and red fluorescent cells, this method allows to quantify NCCR-derived fluorescence/activity without the bias of transfection efficiency and anti-proliferative effects of the tested agents (e.g., dual mTOR inhibitors INK128 or PP242)[5]. Another application of the reporter system is the possibility of analyzing the consequences of insertions, deletions, or duplications in the NCCR found in clinical isolates on the early and late promotor activity. In previous studies, immunosuppressive agents have been studied to modulate NCCR activity; however, no mutations or NCCR rearrangements were found that affect the susceptibility. However, mutations may affect the response of new substances that might be approved in the near future.

This might be relevant since in contrast to the coding regions large T antigen or VP1 capsid protein, the NCCR as the name implies represents a noncoding region and thus does not underlie selective pressure at the amino acid level.

In this protocol, the selection of positive clones containing the NCCR is verified by AgeI and SpeI digestion. However, a colony PCR might represent an alternative approach to quickly screen for NCCR inserts directly from *E. coli* plated on agar plates. For this approach, use primer pair BKV-CR-2-fw-AgeI and EGFP-N (**Table 1**). After transfection, cells are checked for red and green fluorescence using fluorescence microscopy (**Figure 4**). Alternatively, cells can be fixed using 3% PFA for 20 min at 22 °C. In this case, wash with PBS, add 0.2% non-ionic detergent in PBS and incubate for 10 min. Fixed cells can then be stained with DAPI in PBS (1 µg/mL) for 10 min at 22 °C and subjected to fluorescence microscopy analysis and visualized with UV light (340-380 nm).

Since both fluorophores harbor the same polyadenylation signals, an effect of polyadenylation efficiency can be out ruled. However, in comparison to eGFP, tdTomato is a dimeric protein which must be correspondingly transcribed into a longer mRNA (coding sequence: 745 versus 1431 bp,

485 respectively). However, since the activity of the promoters from the cells treated with interfering
486 substances are always compared relative to untreated (DMSO) cells, this difference plays little
487 role. Due to the presence of the origin of replication, the transfection of the reporter plasmid in
488 cells stably expressing the SV40PyV large T antigen allows the transfer of plasmids to the
489 daughter cells. It has been shown that the SV40 derived large T antigen can transactivate SV40
490 and BKPyV NCCR and viral DNA replication[20]. However, since a large T antigen is also involved in
491 the transition from the early to the late phase of infection, an artificial situation is given. Using
492 this assay, it must be considered that the most replication limiting step is the early expression
493 leading to the expression of large T antigen. In order to map the complete replication cycle the
494 early and late mRNAs should be measured in infected cells by qRT-PCR as described elsewhere[5,12].
495
496 A comparable method has already been used by Swiss and German groups to analyze
497 archetypical and rearranged BKPyV NCCR-derived promotor activities, albeit with independently
498 cloned vector systems[5,9]. Gosert and colleagues from Basel used a similar method to determine
499 the NCCR promoter activity of BKPyV and JCPyV strains[9-11]. Both methods used eGFP for green
500 fluorescence; however, the Basel group used RFP while in the current protocol tdTomato was
501 used for red fluorescence. The advantage of tdTomato over RFP is the much higher
502 photostability; however, the dimeric protein is encoded by a sequence that is twice as long as
503 RFP[21]. Furthermore, the half-lives of the proteins might be different, resulting in unequal
504 concentration 72 h post transfection. However, this issue might only be relevant when directly
505 comparing both fluorescence intensities. Gosert et al. used BamHI and NotI as restriction sites
506 while in the current protocol AgeI and SpeI are flanking the NCCR sequence. Using both methods,
507 the reference DUNLOP strain[16] yielded comparable fluorescence patterns with strong early but
508 weak late promoter activity[9,10]. In further agreement, NCCR-rearrangements with insertions in
509 the P-region resulted in a significant increase in early and late promoter activity when compared
510 to wt-NCCR[5,9].
511
512 Although it has been shown that the SV40 derived large T antigen can transactivate SV40 and
513 BKPyV derived NCCRs[20], it might be interesting to use human cells that stably express the large T
514 antigen of the BKPyV and not the prototype virus SV40 in future studies. However, in this case
515 the cell line has to be easy to transfect (e.g., HEK293).
516
517 Since the NCCR promoters of polyomaviruses are very similar, this method can also be used (with
518 small adaptation in the primer sequences) to investigate promoter activity and the influence of
519 potent antiviral agents on the activity of JC-polyomavirus (JCPyV) derived non coding control
520 regions[11]. Since JCPyV is the causative agent of the progressive multifocal leukoencephalopathy
521 (PML), a rare central nervous system disease that can rarely occur in patients with
522 immunodeficiencies resulting in severe neuropathology, there is also a pressing need to find
523 direct acting antiviral substances to treat immune compromised patients. Interestingly,
524 rearrangements are also found in JC NCCRs[11]. Since JCPyV has pronounced neurotropism and
525 thus large quantities of viruses are found in liquor, sample acquisition has to be adjusted to
526 liquor-derived DNA. However, subsequent steps can be easily adapted.
527
528 **ACKNOWLEDGMENTS:**

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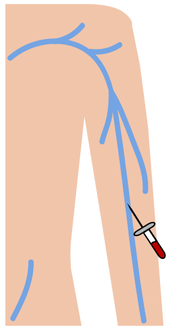
1 sample acquisition



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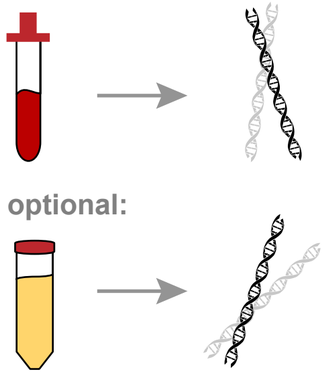

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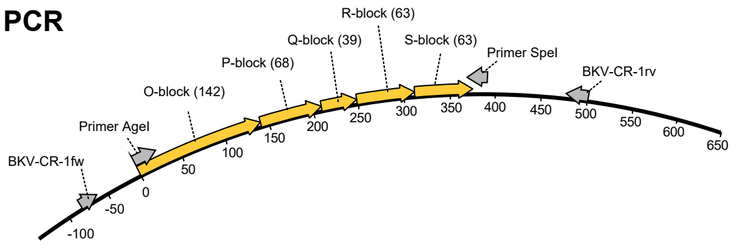

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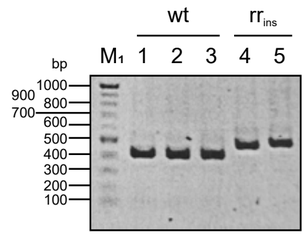

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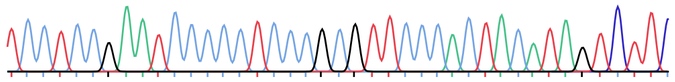

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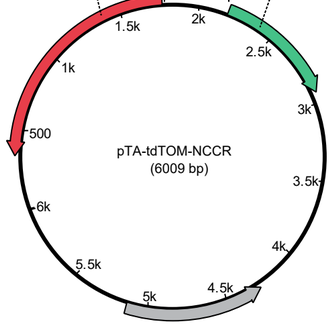

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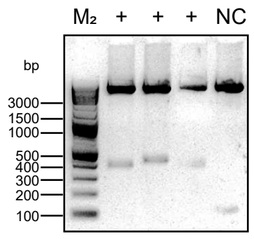

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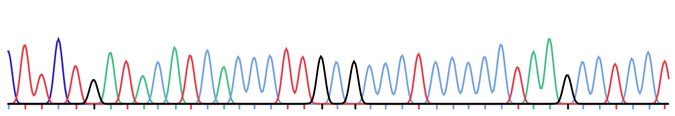

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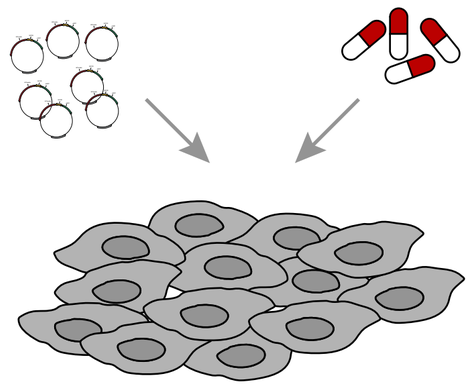

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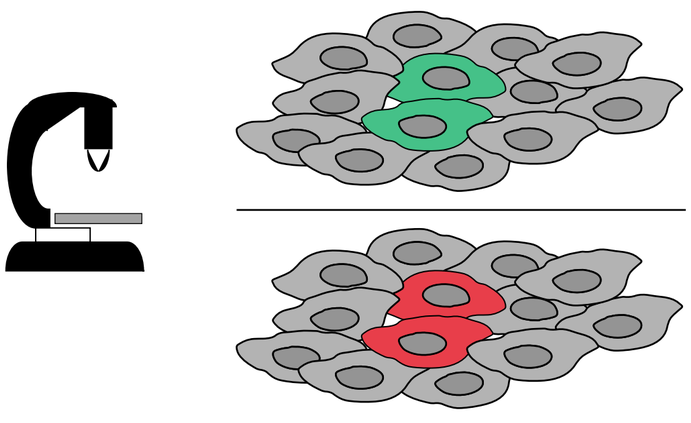

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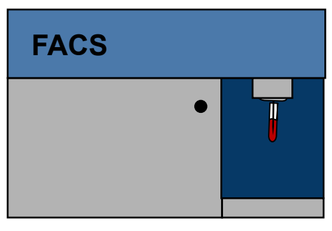

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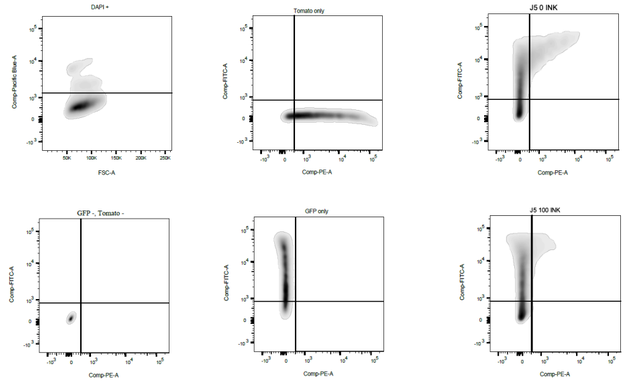

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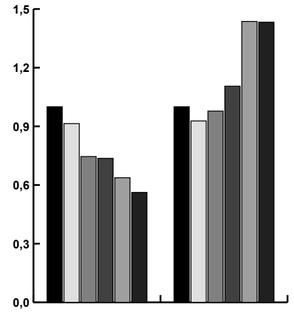

revised figure 3

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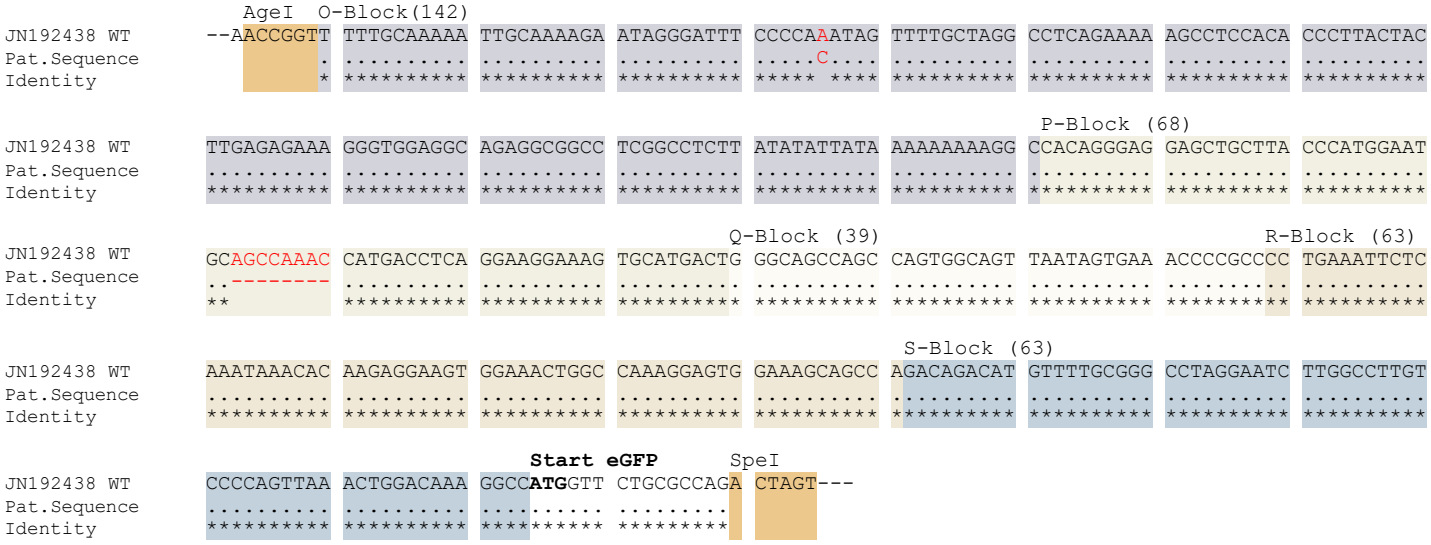

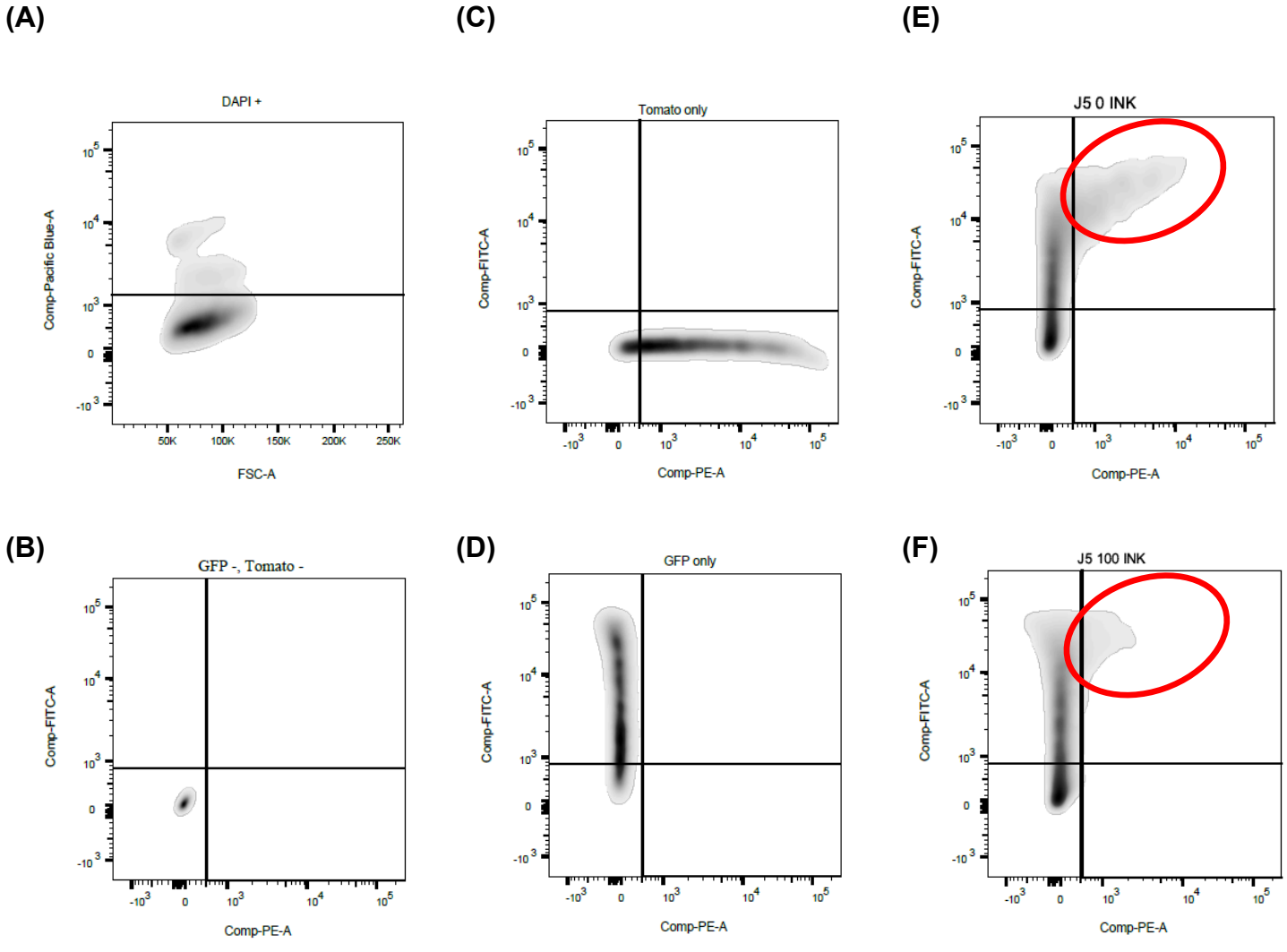

re-revised figure 2

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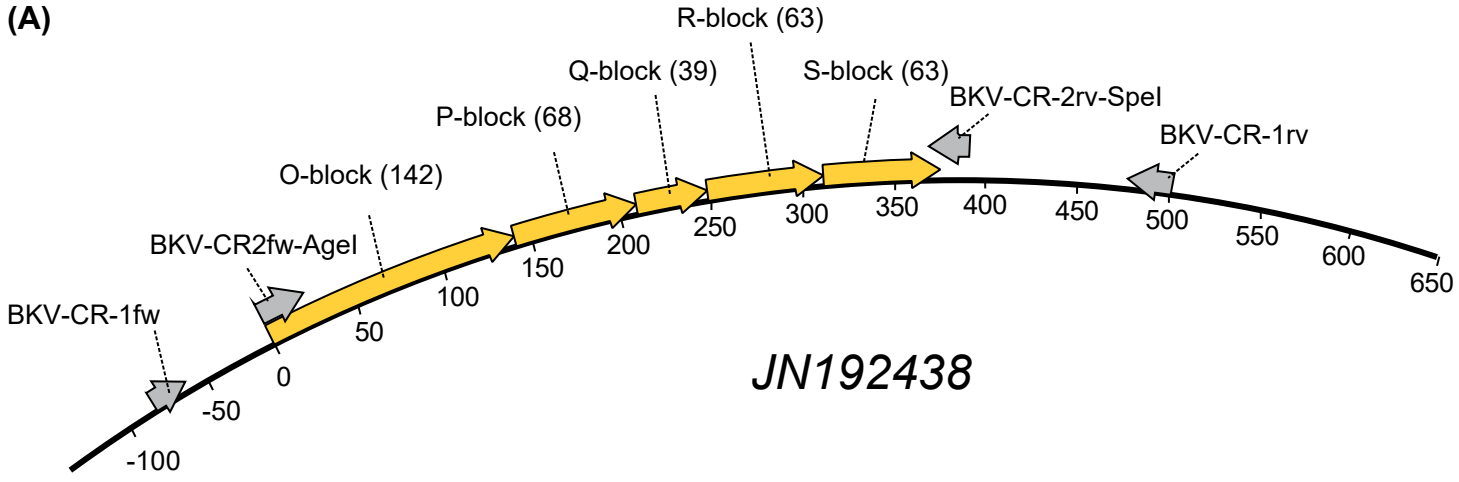

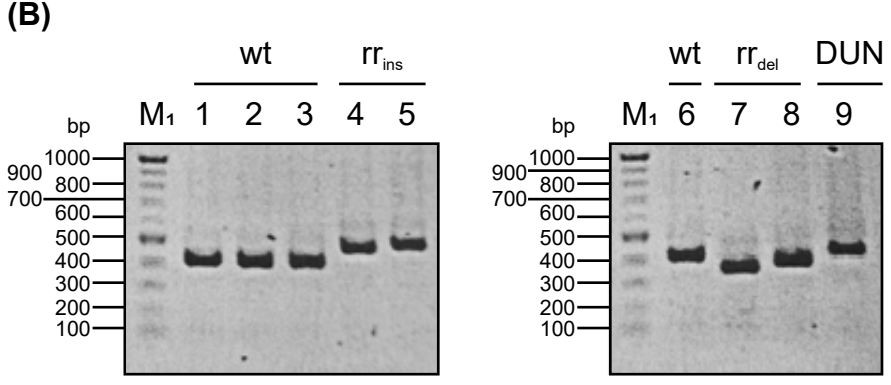

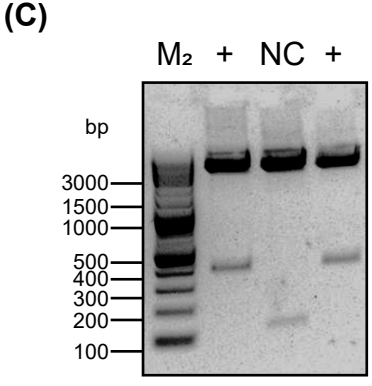

re-revised figure 4

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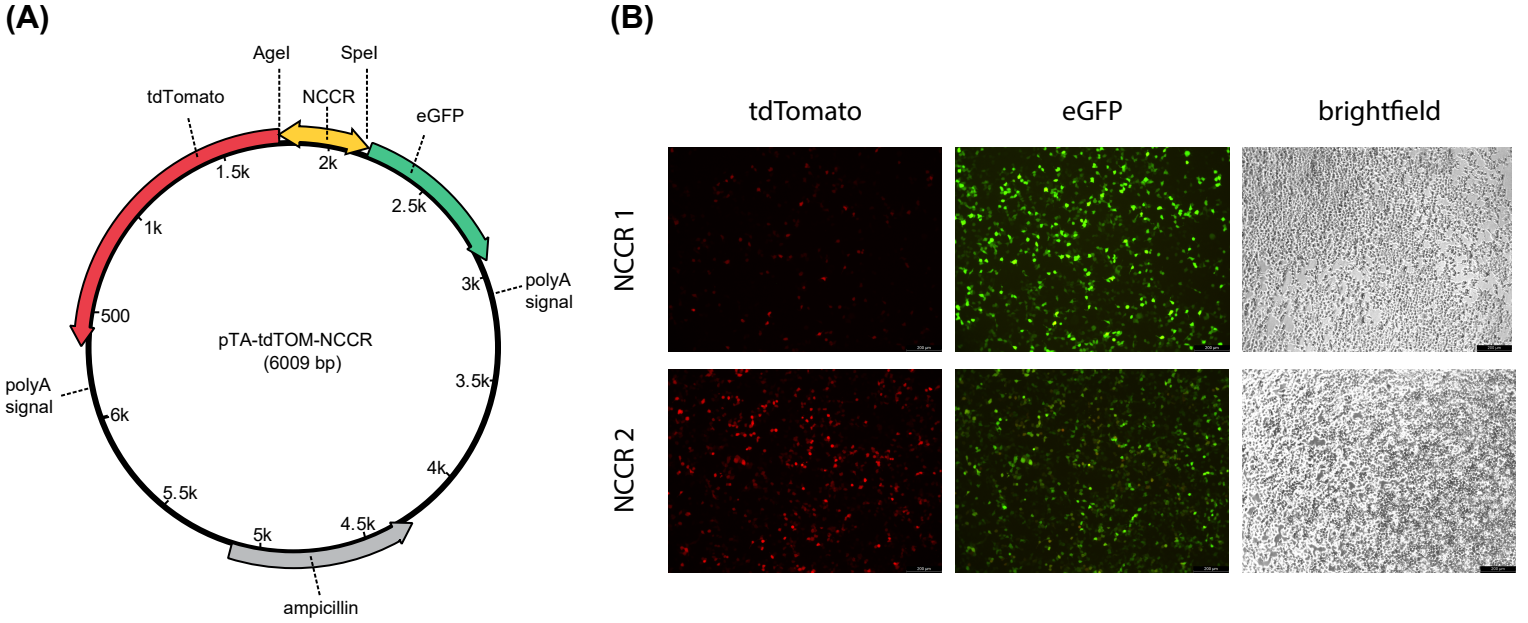

table 1 - primers

Click here to access/download;Table;table1_primers.xlsx

Type of PCR Primer	Primer name	Sequence (5' → 3')
A forward primer	BK-CR-fwd1	CCCAGGCAGCTCTTTCAAGGC
A reverse primer	BK-CR-rev1	CCTCTAACAAAATTCCAGCAAAAGC
B forward primer	BKV-CR-2-fw-AgeI	AAAAAA**ACCGGT**TTTTGCAAAAATTGCAAAAGAATAGG
B reverse primer	BKV-CR-2-rv-SpeI	TTTTTT**ACTAGT**CTGGCGCAGAACCATGGCCTT
Sequencing primer	EGFP-N	CGTCGCCGTCCAGCTCGACCAG

table 2 - PCR Mix

Component	Volume/reaction (µl)	final concentration
RNase-free water	32.8 µL	-
10x PCR Buffer	5 µL	1x
dNTPs (10 mM of each)	1 µL	0.2 mM of each dNTP
Fwd-primer (10 µM)	3 µL	0.3 µM
Rev-primer (10 µM)	3 µL	0.3 µM
Taq Polymerase	0.2 µL	2 unit/reaction
Template DNA	5 µL	<0.5 µg/50 µL reaction

Temperature	Duration	PCR-step	cycles
95°C	5 min	Initial Denaturation	
95°C	30 sec	Denaturation	x35
55°C	34 sec	Annealing	
72°C	1 min	Extension	
72°C	10 min	Final extension	
4°C	∞	Cooling	

table 4 - amplicon digestion

Click here to access/download;Table;table4_amplicon digestion.xlsx

Reagent	Volume/reaction (µl)	final concentration
DNase-free H20	6	to 20 µl
10x Buffer	2	1x
AgeI	1	10 units
SpeI	1	10 units
purified PCR-amplicon	10	variable

table 5 - plasmid digestion

Click here to access/download;Table;table5_plasmid_digestion.xlsx

Reagent	Volume/reaction (µl)	final concentration
DNase-free H20	14.5	to 20 µl
10x Buffer	2	1x
AgeI	1	10 units
SpeI	1	10 units
Plasmid backbone (1 µg/µL)	1.5	1.5 µg

table 6 - ligation protocol

Reagent	Volume/reaction (µl)	final concentration
DNase-free H20	7	to 20 µl
T4 DNA ligase	1	20
10x T4 DNA ligase buffer	2	1x
Purified Plasmid backbone	2	variable
Low melt diluted ½ from step 3.5		
Digested and purified PCR-amplicon from step	8	variable

Name of Material/ Equipment	Company	Catalog Number
100 bp ladder	NEB	N3231
2-log ladder	NEB	N0550
Agar-Agar, Kobe I	Carl Roth	5210.3
AgeI-HF	NEB	R3552
Ampicillin Natriumsalz Cellpure	Carl Roth	HP62.1
Aqua ad iniectabilia	Bbraun	2351744
BD FACSCanto™ II	BD Biosciences	
BD FACSDiva	BD Biosciences	
DAPI	Sigma	10236276001
DFC450C camera module	Leica	
DMEM	Gibco	41966-029
DMIL LED microscope	Leica	
DNA Blood Mini Kit	Qiagen	51104
E.coli DH5alpha Competent Cells	Thermo Scientific	18258012
FBS Superior	MerckMillipore	50615
FlowJo v10.5.3	FlowJo, LLC	
Gel Loading Dye, Purple (6X)	NEB	B7024
HEK293T cells	ATCC	11268
HERAcell® 240i CO2 Incubator	Thermo Scientific	
HotStar PCR kit	Qiagen	203203
Intas Gel documentation system	Intas	
Low Melt Agarose	Biozym	850081
Opti-MEM	Invitrogen	31985070
pBKV (34-2)	ATCC	45025
PBS	Gibco	14190-136
PCR Cycler MJ Mini 48-Well Personal Cycler	Bio-Rad	discontinued product
PCR Nucleotide Mix, 10 mM	Promega	#C1145
PCR1 and PCR2 Primers	metabion	not applicable
PenStrep (100x)	Gibco	15140-122
Roti®-GelStain	Carl Roth	3865

SpeI-HF	NEB	R3133
T4-DNA Ligase	NEB	M0202
TransIT LT1	Mirus	MIR2300
Trypsin 0.05% - EDTA	Gibco	25300-054
ZymoPURE II Plasmid Kit	Zymo	D4201

Comments/Description

Any ladder with a range up to 1 kb can be substituted

Any ladder with a range up to 10 kb can be substituted

can be substituted by any manufacturer

Any camera can be used

can be substituted by any manufacturer

Any fluorescence microscope can be used

can be substituted by any manufacturer

Any FBS can be used

Any flow cytometry software FBS can be used

Any 6x loading dye can be substituted

These cells constitutively express the simian virus 40 (SV40) large T antigen, and clone 17 was selected specifically for its high transfect

can be substituted by any manufacturer

Any visualisation system for stained DNA containing agarose gels can be used

can be substituted by any manufacturer

can be substituted by any manufacturer

Plasmid harboring the full-length genome of BKPyV strain Dunlop; was used as a positive control; DNA Seq. Acc.: KP412983

Any thermocycler can be used

can be substituted by any manufacturer

Desalted. Dilute to (10 µM) with PCR grade water

can be substituted by any manufacturer

A fluorescence based stain for measuring dsDNA concentration

can be substituted by any manufacturer
can be substituted by any manufacturer

:ability.

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Dear Dr. Bajaj,

Thank you again for your critical study of our manuscript. We made several revisions to increase the quality of our article. We hope you will find our second revisions suitable to merit publication of our video and manuscript in *JoVE.*

Point-by-Point Response

Please find the appropriate changes in the revised manuscript highlighted in the Track change mode. We have addressed the following editorial comments in the manuscript:

At least 6-12 keywords are required. Also we cannot have parenthesis in the keyword. So, I have separated the abbreviations. Please check.
> We checked the edited keywords and added new.

The current summary is more than 50-word limit. Please adjust to fit required word numbers. Maybe remove the first sentence?
> We revised the summary and now do not exceed 50 words.

What kind of patient are used? What kind of patients are used in the study?
> We now shortly defined our patient cohort, referring to the previously published study described in Korth et al. 2018.

Please refer to the citations in order of numbering. So, 1 will be cited before 2 or 3.
> We corrected the order of numbering.

Citation for the same? Include citations where this is used for polyoma virus studies.
> We added the citation, accordingly.

Please refer the figures in the order of numbering as well. So figure 1 will be referred before figure 2.
> Figure 1 is now referred before figure 2.

Next day? Please proofread the manuscript well.
> We added additional information (incubation over night).

Citations? (3.10 Isolate the plasmid-DNA using standard protocols),
> We added a citation for a standard protocol for plasmid DNA isolation.

Include the ….treatment with potential antiviral agent as well as shown in the video.
> We now corrected the subtitle, accordingly.

Include the concentration for each of the agents. Also in the introduction, please include why this was used and ciations if any.
> We now included the concentration used in this experiment. We also expanded the introduction, accordingly.

Citation? (Dunlop Strain)
> We now added a citation for the BKPyV-Lab strain Dunlop.

Since you are describing your result, please do not use present tense.
> We proof read the manuscript and do not use present tense any more.

Is this agarose gel pic? There seen to be a white blob on the 4th lane around 1500 bp. Please present a better gel pic.
> We replaced the gel pic with another one without “a white blob”..

Citation for this claim.
> We added a citation

Early or late expression?
> We added the missing information.

Please rewrite this part as if you are describing the result of your experiment where the above protocol was used. E.g. we dis xxx and observed xxx. This leads us to conclude xxx

> We rephrased the part now describing the results of our experiments. We corrected the video voice-over accordingly.

Nowhere in the protocol or introduction there is a mention of this. What kind of patients are used for the study?
> See above

Please include brightfield images of the cells as well. Please also include a scale bar in the figure and describe it in the legend here.

> We replaced the microscopy pictures with those containing a brightfield image and also included a scale bar. The figure legend was revised accordingly.

Video:
> According to the editorial comments concerning the figure quality, we now also included new figures in the manuscript and in the video.

1. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. No need for imperative tense in this section.
> We now revised the voice-over and now describe the experimentally obtained results, which lead to the conclusion that strong and moderate gene expression of NCCRs can be evaluated using this method. Furthermore, we explain that the addition of mTOR-inhibitors reduces red fluorescence and explain why this indicates a reduction in BKPyV early gene expression. In addition, we avoided imperative tense in this section.

2. Please include the title card at the end of the video (after conclusion section) as well
> We included a title card at the end of the video.