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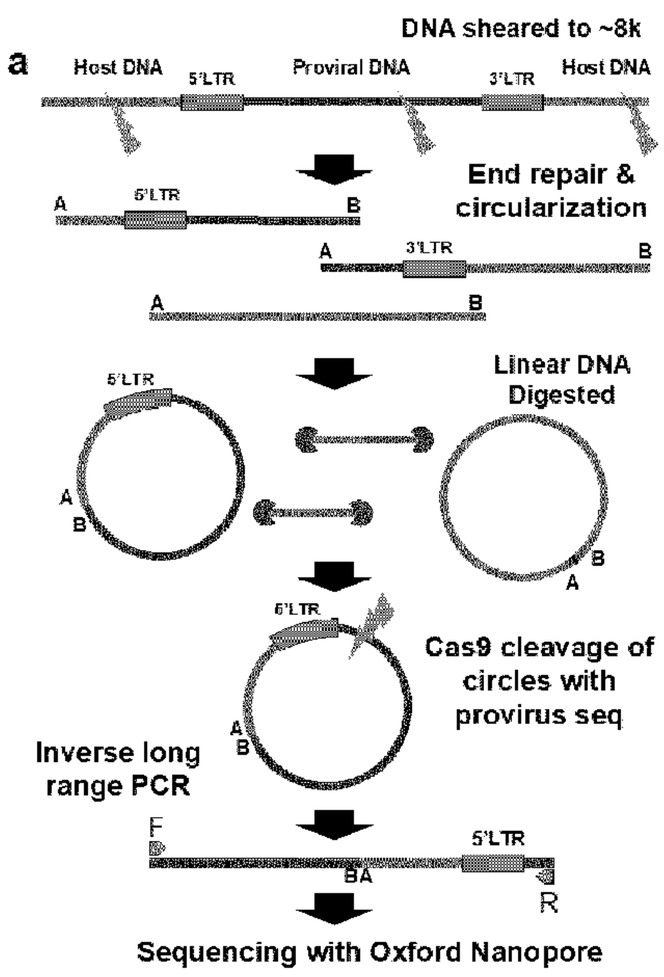
(54) **Title:** POOLED CRISPR INVERSE PCR SEQUENCING (PCIP-SEQ): SIMULTANEOUS SEQUENCING OF VIRAL INSERTION POINTS AND THE INTEGRATED VIRAL GENOMES WITH LONG READS

FIG. 1



(57) **Abstract:** The present invention relates to a method for detecting an integration pattern of a virus, such as human papilloma virus (HPV) in a host genome. In particular, a method is provided encompassing selective cleavage of circularized DNA fragments carrying viral DNA with an RNA-guided endonuclease and at least one guide RNA or at least one pool of guide RNAs, followed by inverse PCR, in particular inverse long-range PCR, and sequencing ("Pooled CRISPR Inverse PCR sequencing (PCIP-seq)". The invention further relates to kits for performing the method and application of the method.

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Pooled CRISPR Inverse PCR sequencing (PCIP-seq): simultaneous sequencing of viral insertion points and the integrated viral genomes with long reads

TECHNICAL FIELD

The present invention relates to a method for detecting an integration pattern of a virus in a host genome, tools for performing the method and applications thereof.

BACKGROUND

The integration of viral DNA into the host genome is a defining feature of the retroviral life cycle, irreversibly linking provirus and cell. This intimate association facilitates viral persistence and replication in somatic cells, and with integration into germ cells bequeaths the provirus to subsequent generations. Considerable effort has been expended to understand patterns of proviral integration, both from a basic virology stand point, and due to the use of retroviral vectors in gene therapy[1]. The application of next generation sequencing (NGS) over the last ~10 years has had a dramatic impact on our ability to explore the landscape of retroviral integration for both exogenous and endogenous retroviruses. Methods based on ligation mediated PCR and Illumina sequencing have facilitated the identification of hundreds of thousands of insertion sites in exogenous viruses such as Human T-cell leukemia virus-1 (HTLV-1)[2] and Human immunodeficiency virus (HIV-1)[3-6]. These techniques have shown that in HTLV-1[2], Bovine Leukemia Virus (BLV)[7] and Avian Leukosis Virus (ALV)[8] integration sites are not random, pointing to clonal selection. In HIV-1 it has also become apparent that provirus integration can drive clonal expansion[3,4,6,9], magnifying the HIV-1 reservoir and placing a major road block in the way of a complete cure.

Current methods based on short-read (high throughput) sequencing identify the insertion point, but the provirus itself is largely unexplored. Whether variation in the provirus influences the fate of the clone remains difficult to investigate. Using long range PCR it has been shown that proviruses in HTLV-1 induced Adult T-cell leukemia (ATL) are frequently (~45%) defective[10], although the abundance of defective proviruses within asymptomatic HTLV-1 carriers has not been systematically investigated. Recently, there has been a concerted effort to better understand the structure of HIV-1 proviruses in the latent reservoir. Methods such as Full-Length Individual Proviral Sequencing (FLIPS) have been developed to identify functional proviruses[11] but without identifying the provirus integration site. More recently matched integration site and proviral sequencing (MIP-Seq) has allowed the

sequence of individual proviruses to be linked to integration site in the genome[6]. However, this method relies on whole genome amplification of isolated HIV-1 genomes, with separate reactions to identify the integration site and sequence the associated provirus[6]. As a result, this method is quite labor intensive limiting the number of proviruses one can reasonably interrogate.

Retroviruses are primarily associated with the diseases they provoke through the infection of somatic cells. Over the course of evolutionary time they have also played a major role in shaping the genome. Retroviral invasion of the germ line has occurred multiple times, resulting in the remarkable fact that endogenous retrovirus (ERV)-like elements comprise a larger proportion of the human genome (8%) than protein coding sequences (~1.5%)[12]. With the availability of multiple vertebrate genome assemblies, much of the focus has been on comparison of ERVs between species. However, single genomes represent a fraction of the variation within a species, prompting some to take a population approach to investigate ERV–host genome variation[13]. While capable of identifying polymorphic ERVs in the population, approaches relying on conventional paired-end libraries and short reads cannot capture the sequence of the provirus beyond the first few hundred bases of the proviral long terminal repeat (LTR), leaving the variation within uncharted.

In contrast to retroviruses, papillomaviruses do not integrate into the host genome as part of their lifecycle. Human papillomavirus (HPV) is usually present in the cell as a multi copy circular episome (~8kb in size), however in a small fraction of infections, it can integrate into the host genome leading to the dysregulation of the viral oncogenes E6 and E7[14]. Genome wide profiling of HPV integration sites via capture probes and Illumina sequencing has also identified hotspots of integration indicating that disruption of host genes may also play a role in driving clonal expansion[15]. As a consequence, HPV integration is a risk factor for the development of cervical carcinoma[16].

HPV accounts for >95% of cervical carcinoma and ~70% of oropharyngeal carcinoma[52]. While infection with a high-risk HPV strain (HPV16 & HPV18) is generally necessary for the development of cervical cancer, it is not sufficient[41]. The progression towards cancer is driven by a combination of both viral and host factors, as a result, a greater understanding of both is required to identify high risk infections[41].

The HPV vaccine will cut the rate of cervical cancer in vaccinated women by ~75%, however it will take 20 to 30 years for the full impact of vaccination to become apparent[64]. Additionally, vaccination uptake varies widely, with the Belgian French speaking community only having a 36% uptake in 2018[65]. As consequence HPV induced cervical cancer will

remain a major health issue in the medium term and the cause of a nontrivial number of cancers into the foreseeable future.

The centrality of HPV integration in carcinogenesis makes a deeper understanding of the process a priority, both to understand the basic biology behind HPV induced cervical cancer, but also because of its potential as a biomarker to identify high risk cases sooner. The study of HPV integration is hampered by the unpredictability of the breakpoint sites in the integrated HPV genome. This limits the applicability of approaches based on ligation mediated PCR and short read sequencing. Techniques such as real-time PCR can identify HPV infections, but cannot identify integrations associated with clonal expansion. Biotin capture probes and Illumina sequencing have provided an unbiased genome wide picture of integration sites in cervical carcinomas, hinting at potential hot spots of integration[15]. However, this technique is not suited to exploring precancerous stages, where only a small fraction of the cells carries integrated virus. Looking beyond integration sites, work on HPV16 using a targeted sequencing approach has shown that conservation of the HPV E7 gene is critical for carcinogenesis[66].

The application of NGS as well as Sanger sequencing before, has had a large impact on our understanding of both exogenous and endogenous proviruses. The development of long-read sequencing, linked-read technologies and associated computational tools[17] have the potential to explore questions inaccessible to short reads. Groups investigating Long interspersed nuclear elements-1 (LINE-1) insertions[18] and the koala retrovirus, KoRV[19] have highlighted this potential and described techniques utilizing the Oxford Nanopore and PacBio platforms, to investigate insertion sites and retroelement structure.

SUMMARY OF THE INVENTION

To more fully exploit the potential of long reads we developed Pooled CRISPR Inverse PCR sequencing (PCIP-seq), a method that leverages selective cleavage of circularized DNA fragments carrying proviral DNA / integrated viral DNA with CRISPR guide RNAs or a pool of CRISPR guide RNAs, followed by inverse long-range PCR and multiplexed sequencing, such as on the Oxford Nanopore MinION platform. Using this approach, we can now simultaneously identify the integration site and track clone abundance while also sequencing the provirus / viral DNA inserted at that position. We have successfully applied the technique to the retroviruses HTLV-1, HIV-1 and BLV, endogenous retroviruses in cattle and sheep as well as HPV18 and HPV16.

In an aspect, the invention provides a method for detecting an integration pattern of human papillomavirus (HPV) in genomic DNA of a subject, said method comprising:

(a) fragmenting genomic DNA isolated from a sample of the subject;

(b) circularizing the DNA fragments to generate circular DNA;

(c) removing non-circularized DNA fragments;

(d) linearizing the circular DNA using an RNA-guided DNA endonuclease and at least one guide RNA or at least one pool of guide RNAs, which target a region in the viral genome, to generate linearized DNA molecules;

(e) amplifying the linearized DNA molecules by an inverse amplification reaction using a pair of primers arranged about and oriented outwardly with respect to the linearization site;

(f) sequencing the amplified DNA;

(g) mapping the sequenced DNA to human genomic DNA sequence; and

(h) optionally mapping the sequenced DNA to the HPV genome.

The invention also provides for a kit for detecting an integration pattern of human papillomavirus (HPV) in genomic DNA of a subject according to the method of of the invention, said kit comprising:

- at least one first guide RNA or at least one first pool of guide RNAs, which target a first region in the viral genome, preferably wherein said first region of the viral DNA comprises E6 gene and/or E7 gene; and/or, preferably and,

-a pair of primers arranged about and oriented outwardly with respect to a first linearization site in the viral genome defined by said at least one first guide RNA or at least first one pool of guide RNAs.

A further aspect relates to a method for monitoring the progression of a human papillomavirus (HPV) infection in a subject comprising:

- detecting an integration pattern of human papillomavirus (HPV) in genomic DNA isolated from a sample of the subject according to the method of the invention; and

- comparing said integration pattern with an integration pattern of HPV in genomic DNA isolated from a sample of the subject at an earlier point in time.

A further aspect relates to a method for assessing a risk of having or developing a cancer in a subject comprising:

- detecting an integration pattern of human papillomavirus (HPV) in genomic DNA of the subject according to the method of the invention; and

- determining whether the integration pattern predisposes the subject to cancer or cancer development. These and further aspects and preferred embodiments of the invention are

described in the following sections and in the appended claims. The subject-matter of the appended claims is hereby specifically incorporated in this specification.

BRIEF DESCRIPTION OF THE FIGURES

The teaching of the application is illustrated by the following Figures which are to be considered as illustrative only and do not in any way limit the scope of the claims.

Figure 1. Overview of the PCIP-seq method **(a)** Simplified outline of method **(b)** A pool of CRISPR guide-RNAs targets each region, the region is flanked by PCR primers. Guides and primers adjacent to 5' & 3' LTRs are multiplexed. **(c)** As the region between the PCR primers is not sequenced we created two sets of guides and primers. Following circularization, the sample is split, with CRISPR mediated cleavage and PCR occurring separately for each set. After PCR the products of the two sets of guides and primers are combined for sequencing. **(d)** Screen shot from the Integrative Genomics Viewer (IGV) showing a small fraction of the resultant reads (grey bars) mapped to the provirus, coverage is shown on top, coverage drops close to the 5' and 3' ends are regions flanked by primers.

Figure 2. PCIP-seq applied to ATL **(a)** In ATL100 both Illumina and Nanopore based methods show a single predominant insertion site **(b)** Screen shot from IGV shows a ~16kb window with the provirus insertion site in the tumor clone identified via PCIP-seq and ligation mediated PCR with Illumina sequencing **(c)** PCIP-seq reads in IGV show a ~3,600bp deletion in the provirus, confirmed via long range PCR and Illumina sequencing. **(d)** The ATL2 tumor clone contains three proviruses (named according to chromosome inserted into), the provirus on chr1 inserted into a repetitive element (LTR) and short reads generated from host DNA flanking the insertion site map to multiple positions in the genome. Filtering out multi-mapping reads causes an underestimation of the abundance of this insertion site (13.6 %), this can be partially corrected by retaining multi-mapping reads at this position (25.4 %). However, that approach can cause the potentially spurious inflation of other integration sites (red slice 9%). The long PCIP-seq reads can span repetitive elements and produce even coverage for each provirus without correction. **(e)** Screen shot from IGV shows representative reads coming from the three proviruses at positions where four *de novo* mutations were observed.

Figure 3. (a) Screen shot from IGV shows representative reads from a subset of the clones from each BLV-infected animal with a mutation in the first base of codon 303 in the viral protein Tax. **(b)** Structural variants observed in the BLV provirus. BLV sense and antisense transcripts are shown on top. Deletions (blue bars) and duplications (red bars) observed in the BLV provirus from both ovine and bovine samples are shown below.

Figure 5. HPV 'looping' integration in an expanded clone **(a)** PCIP-seq reads mapping to a ~87 kb region on chr3 revealed three HPV-host breakpoints. The large number of reads suggests expansion of the clone carrying these integrations. **(b)** PCR was carried out with primer pairs matching regions α and β, as well as α and γ. Both primer pairs produced a ~9kb PCR product. Nanopore sequencing of the PCR products show the HPV genome connects these breakpoints. **(c)** Schematic of the breakpoints with the integrated HPV genome. This conformation indicates that this dramatic structural rearrangement in the host genome was generated via 'looping' integration of the HPV genome.

Figure 6. (a) Reads from four HPV16 samples mapped to the HPV16 subtype A1 genome. Vertical lines identify position where the base differs from the reference genome. (b) Consensus sequences were generated for 12 HPV16 samples and a phylogenetic tree with the HPV16 subtype reference genomes (highlighted) was generated. The 12 samples cluster with the HPV16 A1 and A2, both are European isolates.

Figure 7. Clone persistence was observed in two patients. The first patient had an integration in the LAPTM4B gene (histology= ASC-H), a second sampling from 7 months later (upgraded to HSIL) showed the same integration sites (a) The discordant breakpoints again points to 'looping' integration in an expanded clone. (b) When the reads are mapped to the HPV genome the sample from July 2019 has reads originating from episomal copies of HPV as well as reads from the integrated copy of HPV. All the HPV reads from the December 2019 sample contain the deletion associated with the integrated copy of HPV indicating that the infection has cleared but the clonally expanded cell remains. PCR with primer pairs matching regions a and 13 produced a ~9kb PCR product, again indicating that the integration has caused a structural rearrangement in this region. (c) In the second patient (a 71 year old, histology= ASC-US at both time points) HPV was found to be integrated at three positions in the genome (within exons of the genes *TMEM177, IL20RB* and *ARMH3*), introducing at least three copies of HPV (E6 and E7 are intact in thep integrated HPV genomes). It is not possible to tell at this point if all are in the same or separate clones. (d) For both time points the integrated HPV reads represent -10% of the total HPV reads, although the greater number of unique shear sites in the second time point (especially for the chr2 integration) suggest the clone may be expanding.

Figure 8. Use of Cas-9 mediated cleavage in the PCIP-seq method. 8 µg of DNA from a BLV infected sheep with a proviral load of 82.6% was circularized and linear DNA was eliminated. One quarter of the resultant DNA was subject to CRISPR-cas9 cleavage using the Pool A guides (CRISPR+, PA), the second quarter was cleaved using the Pool B guides (CRISPR+, PB), the remaining half was kept aside. The linearized DNA was cleaned and

used as template in 2x 50µl PCR reactions using the appropriate primer pairs for Pool A (PA) or Pool B (PB). For the uncut DNA half was used as template for 2x 50µl PCR reactions using the PA primers (CRISPR-, PA) and the other half was used for 2x 50µl PCR reactions using the PB primers (CRISPR-, PB). Following 25 PCR cycles, 10µl of each reaction were loaded on a 1% agarose gel. A=unshared genomic DNA, B=genomic DNA sheared to 8kb.

Figure 9. Coverage of the pure viral reads as well as the chimeric reads produced by the libraries shown in Figure 8 on the BLV proviral genome. BC refers to the barcode used for each library.

Figure 10. Pie charts showing the relative abundance of the 200 largest clones in the four sheep (top) and three cattle (bottom) infected with BLV, each slice of the pie represents a single insertion site, the % below indicated what fraction of the overall reads these 200 clones represent.

DESCRIPTION

As used herein, the singular forms “a”, “an”, and “the” include both singular and plural referents unless the context clearly dictates otherwise.

The terms “comprising”, “comprises” and “comprised of” as used herein are synonymous with “including”, “includes” or “containing”, “contains”, and are inclusive or open-ended and do not exclude additional, non-recited members, elements or method steps. The terms also encompass “consisting of” and “consisting essentially of”, which enjoy well-established meanings in patent terminology.

The recitation of numerical ranges by endpoints includes all numbers and fractions subsumed within the respective ranges, as well as the recited endpoints. This applies to numerical ranges irrespective of whether they are introduced by the expression “from… to…” or the expression “between… and…” or another expression.

The terms “about” or “approximately” as used herein when referring to a measurable value such as a parameter, an amount, a temporal duration, and the like, are meant to encompass variations of and from the specified value, such as variations of +/-10% or less, preferably +/-5% or less, more preferably +/-1% or less, and still more preferably +/-0.1% or less of and from the specified value, insofar such variations are appropriate to perform in the disclosed invention. It is to be understood that the value to which the modifier “about” or “approximately” refers is itself also specifically, and preferably, disclosed.

Whereas the terms “one or more” or “at least one”, such as one or more members or at least one member of a group of members, is clear per se, by means of further

exemplification, the term encompasses inter alia a reference to any one of said members, or to any two or more of said members, such as, e.g., any ≥3, ≥4, ≥5, ≥6 or ≥7 etc. of said members, and up to all said members. In another example, “one or more” or “at least one” may refer to 1, 2, 3, 4, 5, 6, 7 or more.

The discussion of the background to the invention herein is included to explain the context of the invention. This is not to be taken as an admission that any of the material referred to was published, known, or part of the common general knowledge in any country as of the priority date of any of the claims.

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. All documents cited in the present specification are hereby incorporated by reference in their entirety. In particular, the teachings or sections of such documents herein specifically referred to are incorporated by reference.

Unless otherwise defined, all terms used in disclosing the invention, including technical and scientific terms, have the meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. By means of further guidance, term definitions are included to better appreciate the teaching of the invention. When specific terms are defined in connection with a particular aspect of the invention or a particular embodiment of the invention, such connotation or meaning is meant to apply throughout this specification, i.e., also in the context of other aspects or embodiments of the invention, unless otherwise defined.

In the following passages, different aspects or embodiments of the invention are defined in more detail. Each aspect or embodiment so defined may be combined with any other aspect(s) or embodiment(s) unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with any other feature or features indicated as being preferred or advantageous.

Reference throughout this specification to “one embodiment”, “an embodiment” means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, appearances of the phrases “in one embodiment” or “in an embodiment” in various places throughout this specification are not necessarily all referring to the same embodiment, but may. Furthermore, the particular features, structures or characteristics may be combined in any suitable manner, as would be apparent to a person skilled in the art from this disclosure, in one or more embodiments. Furthermore, while some embodiments described herein

include some but not other features included in other embodiments, combinations of features of different embodiments are meant to be within the scope of the invention, and form different embodiments, as would be understood by those in the art. For example, in the appended claims, any of the claimed embodiments can be used in any combination.

For general methods relating to the invention, reference is made inter alia to well-known textbooks, including, e.g., "Molecular Cloning: A Laboratory Manual, 4th Ed." (Green and Sambrook, 2012, Cold Spring Harbor Laboratory Press), "Current Protocols in Molecular Biology" (Ausubel et al., 1987).

Provided herein is a method for detecting an integration pattern of a virus in genomic DNA of a subject, said method comprising:

(a) fragmenting genomic DNA isolated from a sample of the subject;

(b) circularizing the DNA fragments to generate circular DNA;

(c) removing non-circularized DNA fragments;

(d) optionally linearizing the circular DNA using an RNA-guided DNA endonuclease and at least one guide RNA or at least one pool of guide RNAs, which target a region in the viral genome to generate linearized DNA molecules;

(e) amplifying the circular DNA or the linearized DNA molecules by an inverse amplification reaction using a pair of primers arranged about and oriented outwardly with respect to the linearization site;

(f) sequencing the amplified DNA;

(g) mapping the sequenced DNA to genomic DNA sequence of the subject; and

(h) optionally mapping the sequenced DNA to the viral genome.

As used herein, the terms "integration pattern" or "viral integration pattern" refer to the pattern of viral DNA that is integrated in host genomic DNA. The term may refer to a visualized DNA pattern comprising viral DNA and host genomic DNA, as well as to information quantified by or correlated with such DNA pattern. Non-limiting examples of information quantified by, or correlated with an integration pattern include the presence of absence of integrated viral DNA; the number of viral integration sites in host genomic DNA or the average number of such integrations; the insertion site(s) of viral DNA in the host genome; mutations (e.g. deletions, duplications, SNPs, etc.) in the viral DNA integrations; the size in kb of viral DNA integrations into host genomic DNA; the number of viral genomes integrated at each integration site; the number of viral integration sites per cellular genome; the mean number of viral genomes integrated per integration site (or the mean size of integration sites); maximum number of viral genomes integrated per integration site (or the maximum size of integration sites); minimum number of viral genomes integrated per

integration site (or minimum size of integration sites), number of viral genomes integrated per cellular genome, and any combinations thereof.

The method of the invention allows to detect integration of viruses such as retroviruses that integrate into a host cell genome as part of their lifecycle, as well as viruses such as papillomaviruses that do not integrate into a host cell genome as part of their lifecycle. The virus may be a DNA virus or an RNA virus. DNA viruses include, for example, human papillomavirus (HPV); RNA viruses include, for example, human T lymphophilic virus (HTLV, particularly HTLV-1), human immunodeficiency virus (HIV), bovine leukemia virus (BLV). In embodiments, the virus is a retrovirus. In further embodiments, the retrovirus is an exogenous retrovirus such as HTLV, in particular HTLV-1, HIV or BLV. In further embodiments, the retrovirus is an endogenous retrovirus. In other embodiments, the virus is HPV. In further embodiments, said HPV is a high risk HPV such as a HPV strain 16, 18, 31, 33, 35, 39, 45, 51, 55, 56, 58, 59 or 66, preferably a HPV strain 18 or a HPV strain 16.

"Integrated viral DNA" refers to a complete or partial genome of a virus that is integrated into a host cell chromosome. "Episomal viral DNA" refers to non-integrated viral DNA, i.e., viral DNA that has not integrated into a host cell chromosome. “Provirus” refers to viral DNA, in particular retroviral DNA, that is integrated into the DNA of a host cell as a stage of virus replication, or a state that persists over longer periods of time as either inactive viral infections or an endogenous viral element.

The terms "subject" and “host” and "patient" are used interchangeably and refer to a human or non-human animal that is tested for the presence of integrated viral DNA. The host is not particularly limited as long as the virus infects and viral nucleic acid is integrated into the genome. Preferably, the host is a mammal, most preferably a human. Hosts may be domestic animals such as cows, horses, pigs, sheep, goats and chickens. In preferred embodiments, the subject is a human. In embodiments, the subject is an ovine. In embodiments, the subject is a bovine.

The term "sample" generally refers to a material of biological origin that includes cells. Samples can include, e.g., an *in vitro* cell culture or tissue obtained from a subject as defined herein. Samples can be purified or semi-purified to remove certain constituents (e.g., extracellular constituents or non-target cell populations). In embodiments, the sample comprises cervical or vaginal epithelial cells, such as wherein the sample is a pap smear. In embodiments, the sample comprises oropharyngeal epithelial cells, such as wherein the sample is an oropharyngeal swab. In embodiments, the sample comprises peripheral blood mononuclear cells (PBMC), in particular CD4+ T cells, such as wherein the sample is a

blood sample, e.g. a whole blood sample. In embodiments, the sample is a sperm sample. Isolation of DNA from the samples can be carried out by standard methods.

In step (a) genomic DNA of the subject is fragmented. In embodiments, fragmenting the genomic DNA of the subject comprises shearing the genomic DNA, thereby producing (sheared) DNA fragments. Shearing of the genomic DNA may occur e.g. by acoustic or mechanical means as known to the skilled person. In further embodiments, shearing of the genomic DNA of the subject is followed by end-repair of the sheared DNA fragments.

In embodiments, the (sheared) DNA fragments have an average size of about the size of the viral genome. In particular embodiments, the (sheared) DNA fragments have an average size of between 6000 and 10000 basepairs (bp), preferably between 7000 and 9000 bp, more preferably about 8000 bp.

In step (b) of the method (sheared) DNA fragments are circularized. Circularization or intramolecular ligation of the DNA fragments may be achieved by incubation of the DNA fragments in the presence of a DNA ligase, e.g. T4 DNA ligase, as known to the skilled person, thereby generating circular DNA.

Step (c) of the method encompasses removal of remaining linear DNA. In embodiments, non-circularized DNA is removed by digestion. Selective digestion of non-circularized or linear DNA may be achieved using an appropriate selective DNase as commercially available (e.g. Plasmid-Safe™ ATP-Dependent DNase (Epicentre).

Preferably, the circular DNA is linearized in step (d) before the amplification step (e), which improves the efficiency of the amplification reactions. Linearization of the circular DNA can be achieved using an RNA-guided DNA endonuclease, such as a CRISPR-Cas system as known to the skilled person, and corresponding guide RNAs. In particular embodiments, the RNA-guided DNA endonuclease is a Cas-9 endonuclease.

In order to achieve selective linearization of circular DNA that comprises integrated viral DNA and host DNA, guide RNA(s) are used that target a region of the viral DNA. Preferably, the “linearization site”, i.e. the region in the viral DNA that is targeted by a guide RNA or a pool of guide RNAs, comprises a region of the viral genome that is prone to integration in host DNA. For example, for HPV, a linearization site may comprise E6 gene and/or E7 gene. For retroviruses, a linearization site may be adjacent to a 5’LTR or adjacent to a 3’LTR.

Particular guide RNA targeting domains and pools of guide RNA targeting domains are provided in Table 1. The sequences set forth in SEQ ID NO:7-79 refer to oligonucleotide sequences used for synthesizing the guide RNAs. These sequences comprise a “targeting

domain" as well as accessory sequences required by the kit, in particular the EnGen® sgRNA Synthesis Kit (New England Biolabs), for synthesizing the guide RNA, which elements can be identified by the skilled person. By way of example, oligonucleotide sequences encoding HPV18 and HPV16 gRNAs and their corresponding targeting domain and flanking PAM site (underlined) are summarized in the below table. With "targeting domain" is meant herein a sequence that is capable of hybridizing to a sequence in the region of the viral DNA that is targeted by the guide RNA (i.e. in the linearization site of the viral DNA). With "PAM site" is meant herein a protospacer adjacent sequence as is known in the art. When reference is made to a guide RNA comprising a sequence set forth in any one of SEQ ID NO:7-79, a guide RNA comprising the targeting domain of said sequence is envisaged, i.e. the sequence without the sequence TTCTAATACGACTCACTATA (SEQ ID NO:244) 5 prime and without the sequence GTTTTAGAGCTAGA (SEQ ID NO:245) 3 prime. When reference is made to a guide RNA comprising a sequence set forth in any one of SEQ ID NO:232-243, a guide RNA comprising the targeting of said sequence is envisaged, i.e. the sequence without the NGG sequence 3 prime. As will be appreciated by the skilled person, the guide RNA comprises in addition to a targeting domain, a tracer and a tracer mate as known in the art, wherein the tracer and tracer mate may be provided chimeric. The guide RNA is an RNA molecule and will therefore comprise the base uracil (U), while the oligonucleotide encoding the gRNA molecule comprises the base thymine (T).

Guide RNA	**Oligonucleotide**	**SEQ ID NO:**	**Targeting domain and flanking PAM site**	**SEQ ID NO:**
HPV18 Region 1 Guide RNA				
1_HPV18_R1_guide1	TTCTAATACGACTCACTATAGTGCTGCA ACCGAGCACGACGTTTTAGAGCTAGA	68	GTGCTGCAACCG AGCACGACAGG	232
2_HPV18_R1_guide2	TTCTAATACGACTCACTATAGTGCTCGG TTGCAGCACGAAGTTTTAGAGCTAGA	69	GTGCTCGGTTGC AGCACGAATGG	233
3_HPV18_R1_guide3	TTCTAATACGACTCACTATAGCGACGAT TTCACAACATAGCGTTTTAGAGCTAGA	70	CGACGATTTCAC AACATAGCTGG	234
HPV18 Region 2 Guide RNA				
8_HPV18_R2_guide4	TTCTAATACGACTCACTATAGATTTTAG AGGATTGGAACTTGTTTTAGAGCTAGA	71	ATTTTAGAGGAT TGGAACTTTGG	235
9_HPV18_R2_guide5	TTCTAATACGACTCACTATAGTCTGCTA TACTGCTTAAATTGTTTTAGAGCTAGA	72	TCTGCTATACTG CTTAAATTTGG	236
10_HPV18_R2_guide6	TTCTAATACGACTCACTATAGCATCATA TTGCCCAGGTACGTTTTAGAGCTAGA	73	GCATCATATTGC CCAGGTACAGG	237
HPV16_E6-E7 Guide RNA				
3261_HPV16_E6-E7_G1	TTCTAATACGACTCACTATAGCTAATTA ACAAATCACACAAGTTTTAGAGCTAGA	74	CTAATTAACAAA TCACACAACGG	238
3262_HPV16_E6-E7_G2	TTCTAATACGACTCACTATAGATTCCAT AATATAAGGGGTGTTTTAGAGCTAGA	75	GATTCCATAATA TAAGGGGTCGG	239
3263_HPV16_E6-E7_G3	TTCTAATACGACTCACTATAGCAACAAG ACATACATCGACGTTTTAGAGCTAGA	76	GCAACAAGACAT ACATCGACCGG	240
HPV16_L1 Guide RNA				

3266_HPV16_L1_G1	TTCTAATACGACTCACTATAGCCACCTA TAGGGGAACACTGGTTTTAGAGCTAGA	77	CCACCTATAGGG GAACACTGGGG	241
3267_HPV16_L1_G2	TTCTAATACGACTCACTATAGACCTACC TCAACACCTACACGTTTTAGAGCTAGA	78	ACCTACCTCAAC ACCTACACAGG	242
3268_HPV16_L1_G3	TTCTAATACGACTCACTATAGTAATAGA GAATGTATATCTAGTTTTAGAGCTAGA	79	TAATAGAGAATG TATATCTATGG	243

To improve cleavage of a linearization site, more than one guide RNA targeting said linearization site can be used. As used herein, a “pool of guide RNAs” refers to a set of guide RNAs that target a defined region of the viral DNA, i.e. the linearization site. It is to be understood that each guide RNA within a pool of guide RNAs may be capable of hybridizing to different, non-overlapping or partially overlapping, sequences within said linearization site. A pool of guide RNAs may comprise at least 2 or at least 3 guide RNAs, preferably at least 3 guide RNAs, more preferably between 3 and 10 or between 3 and 8 guide RNAs, such as 3, 4, 5, 6, 7 or 8 guide RNAs.

The circular DNA may be linearized using a first guide RNA or a first pool of guide RNAs, which target a first region of the viral DNA, and at least one other guide RNA or at least one other pool of guide RNAs, which target a non-overlapping region(s) of the viral RNA. When targeting more than one linearization site, a more complete integration pattern may be obtained (e.g. more integration sites may be detected).

Accordingly, in embodiments, a first portion of the circular DNA is linearized using a first guide RNA or a first pool of guide RNAs that target a first region of the viral DNA to generate a first set of linearized DNA molecules; and

a second portion of the circular DNA is linearized using a second guide RNA or a second pool of guide RNAs that target a second region of the viral DNA to generate a second set of linearized DNA molecules,

wherein the first region and the second region of the viral DNA do not overlap.

In embodiments of the method for detecting an integration pattern of a retrovirus in genomic DNA of a subject, a first portion of the circular DNA is linearized using a first guide RNA or a first pool of guide RNAs that target a region of the viral DNA adjacent to the 5’ long terminal repeat (LTR) to generate a first set of linearized DNA molecules; and

a second portion of the circular DNA is linearized using a second guide RNA or a second pool of guide RNAs that target a region of the viral DNA adjacent to the 3’LTR to generate a second set of linearized DNA molecules.

In embodiments of the method for detecting an integration pattern of a HPV in genomic DNA of a subject, a first portion of the circular DNA is linearized using a first guide RNA or a first pool of guide RNAs that target a first region of the viral DNA comprising E6 gene and/or E7 gene to generate a first set of linearized DNA molecules; and

a second portion of the circular DNA is linearized using a second guide RNA or a second pool of guide RNAs that target a second region of the viral DNA to generate a second set of linearized DNA molecules, wherein said first and second regions of the viral DNA do not overlap.

In the amplification step (e), the circular DNA or preferably the linearized DNA molecules are amplified by an inverse amplification reaction using a pair of primers arranged about and oriented outwardly with respect to the linearization site. In particular, a primer pair is used comprising a forward primer capable of hybridizing to a viral DNA sequence in a 3' flanking region of the viral DNA region targeted by the guide RNA or the pool of guide RNAs and a reverse primer capable of hybridizing to a viral DNA sequence in a 5' flanking region of the viral DNA region targeted by the guide RNA or the pool of guide RNAs.

Particular primer pairs corresponding to the guide RNA targeting domains or pools of guide RNA targeting domains of Table 1 are provided in Table 2. The primers in Table 2 may comprise a tail, in particular a tail consisting of the sequence TTTCTGTTGGTGCTGATATTGC (SEQ ID NO:246) or the sequence ACTTGCCTGTCGCTCTATCTTC (SEQ ID NO:247). When reference is made herein to a primer comprising a sequence set forth in any one of SEQ ID NO:80-127, the tailed primer as well as a corresponding primer without the tail or with another tail are envisaged herein.

Preferably, each set of linearized DNA molecules (i.e. linearized DNA molecules generated by one guide RNA or one pool of guide RNAs as described herein and thus characterized by cleavage in a defined linearization site) is amplified in a separate amplification reaction using an appropriate pair of primers arranged about and oriented outwardly with respect to the linearization site.

In further embodiments, the linearization step and the amplification step may be carried out in a single solution, wherein a guide RNA or a pool of guide RNAs and a corresponding pair of primers are multiplexed.

In preferred embodiments, said amplification reaction comprises a long range amplification reaction such as a long range PCR. As used herein, "long range PCR" refers to a method to amplify DNA fragments of increased size, typically of more than 3-5 kb, using a modified DNA polymerase or high-fidelity DNA polymerase. DNA polymerases for long range PCR are known to the skilled person and are commercially available.

In further embodiments, tailed primers are used in the amplification reaction and the amplicons are subjected to a second amplification reaction using a set of indexing primers,

thereby generating indexed amplification products. This facilitates multiplexed sequencing of the amplified DNA.

Particular methods are provided herein for detecting an integration pattern of a retrovirus in genomic DNA of a subject, said method comprising:

(a) fragmenting genomic DNA isolated from a sample of the subject;

(b) circularizing the DNA fragments to generate circular DNA;

(c) removing non-circularized DNA fragments;

(d) linearizing the circular DNA using an RNA-guided DNA endonuclease and at least one guide RNA or at least one pool of guide RNAs, which target a region in the viral genome adjacent to the 5' long terminal repeat (LTR) or adjacent to the 3'LTR to generate linearized DNA molecules;

(e) amplifying the linearized DNA molecules by an inverse amplification reaction using a pair of primers arranged about and oriented outwardly with respect to the linearization site;

(f) sequencing the amplified DNA;

(g) mapping the sequenced DNA to genomic DNA sequence of the subject; and

(h) optionally mapping the sequenced DNA to the viral genome.

In further embodiments of the method for detecting an integration pattern of a retrovirus in genomic DNA of a subject, the linearization of the circular DNA comprises linearizing a first portion of the circular DNA using a first guide RNA or a first pool of guide RNAs, preferably a first pool of guide RNAs, which target a region of the viral DNA adjacent to the 5' long terminal repeat (LTR) to generate a first set of linearized DNA molecules, and

linearizing a second portion of the circular DNA using a second guide RNA or a second pool of guide RNAs, preferably a second pool of guide RNAs, which target a region of the viral DNA adjacent to the 3'LTR to generate a second set of linearized DNA molecules; and

the amplification of the linearized DNA molecules comprises amplifying the first set of linearized DNA molecules using a first pair of primers arranged about and oriented outwardly with respect to the viral DNA region adjacent to the 5' LTR targeted by the first guide RNA or the first pool of guide RNAs,

and amplifying the second set of linearized DNA molecules using a second pair of primers arranged about and oriented outwardly with respect to the viral DNA region adjacent to the 3' LTR targeted by the second guide RNA or the second pool of guide RNAs.

A further aspect relates to a kit for performing the method described herein, said kit comprising:

- at least one first guide RNA or at least one first pool of guide RNAs, which target a first region of the viral DNA; and/or, preferably and,
- a pair of primers arranged about and oriented outwardly with respect to a first linearization site in the viral DNA defined by said at least one first guide RNA or at least one first pool of guide RNAs.

In further embodiments, the kit comprises:

- a first guide RNA or a first pool of guide RNAs, which target a first region of the viral DNA;
- a second guide RNA or a second pool of guide RNAs, which target a second region of the viral DNA, wherein the first and the second regions of the viral DNA do not overlap;
- a first pair of primers arranged about and oriented outwardly with respect to a first linearization site in the viral DNA defined by said first guide RNA or said first pool of guide RNAs; and/or, preferably and,

a second pair of primers arranged about and oriented outwardly with respect to a second linearization site in the viral DNA defined by said second guide RNA or said second pool of guide RNAs.

Particular kits are provided herein for the detection of an integration pattern of a HPV in genomic DNA of a subject according to the method disclosed herein, said kit comprising:

- at least one guide RNA or at least one pool of guide RNAs, which target a region of the viral DNA comprising E6 gene and/or E7 gene; and/or, preferably and
- a pair of primers arranged about and oriented outwardly with respect to a linearization site in the viral DNA defined by said at least one guide RNA or at least one pool of guide RNAs.

In other embodiments, said kit comprises:

- at least one guide RNA or at least one pool of guide RNAs, which target a region of the viral DNA comprising or adjacent to L1 gene; and
- a pair of primers arranged about and oriented outwardly with respect to a linearization site in the viral DNA defined by said at least one guide RNA or at least one pool of guide RNAs.

In further embodiments, said kit for the detection of an integration pattern of a HPV comprises:

- a first guide RNA or a first pool of guide RNAs, which target a first region of the viral DNA comprising E6 gene and/or E7 gene;
- a first pair of primers arranged about and oriented outwardly with respect to a linearization site in the viral DNA defined by said first guide RNA or said first pool of guide RNAs;

- a second guide RNA or a second pool of guide RNAs, which target a second region of the viral DNA, wherein said first and second regions of the viral DNA do not overlap; and

- a second pair of primers arranged about and oriented outwardly with respect to a linearization site in the viral DNA defined by said second guide RNA or said second pool of guide RNAs.

In particular embodiments, said second region of the viral DNA comprises a region of the viral DNA comprising L1 gene or a region of the viral DNA adjacent to L1 gene.

Particular embodiments for the guide RNAs, pools of guide RNAs and primer pairs are as described above for the method. Particular combinations of guide RNA targetind domains or pools of guide RNA targeting domains and primer pairs are described in Tables 1 and 2.

The kit may also contain reagents, e.g., buffers, enzymes and other necessary reagents, for performing the method described above. In particular embodiments, the kit further comprises an RNA-guided DNA endonuclease. In particular embodiments, the kit further comprises a DNA polymerase, preferably a DNA polymerase for long range PCR.

The various components of the kit may be present in separate containers or certain compatible components may be pre-combined into a single container, as desired.

The herein disclosed aspects and embodiments of the invention are further supported by the following non-limiting examples.

EXAMPLES

Example 1: Materials and methods

Samples

Both the BLV infected sheep[7] and HTLV-1 samples[7,20] have been previously described. Briefly, the sheep were infected with the molecular clone pBLV344[21], following the experimental procedures approved by the University of Saskatchewan Animal Care Committee based on the Canadian Council on Animal Care Guidelines (Protocol #19940212). The HTLV-1 samples[7,20] were obtained with informed consent following the institutional review board-approved protocol at the Necker Hospital, University of Paris, France, in accordance with the Declaration of Helsinki. The BLV bovine samples were natural infections, obtained from commercially kept adult dairy cows in Alberta, Canada. Sampling was approved by VSACC (Veterinary Sciences Animal care Committee) of the University of Calgary: protocol number: AC15-0159. The bovine 571 used for ERV identification was collected as part of this cohort. The two sheep samples used for Jaagsiekte sheep retrovirus (enJSRV) identification were the BLV infected ovine samples

(220 & 221 (032014)), with a PVL of 3.8 and 16% respectively. PBMCs were isolated using standard Ficoll-Hypaque separation. The DNA for the bovine Mannequin was extracted from sperm, while the DNA for bovine 10201e6 was extracted from whole blood using standard procedures. The HIV-1 U1 cell line DNA sequenced without dilution was provided by Dr. Carine Van Lint, IBMM, Gosselies, Belgium. The HIV-1 U1 cell line dilutions in Jurkat were generated at Ghent University Hospital.

HPV material was prepared from PAP smears obtained from HPV-infected patients at the CHU Liège University hospital. Both patients were PCR positive for HPV18, HPV18_PY was classified as having Atypical Squamous Cell of Undetermined Significance (ASC-US), while HPV18_PX was classified as having Atypical Glandular Cells (AGC). Patients provided written informed consent and the study was approved by the Comité d'Ethique Hospitalo-Facultaire Universitaire de Liège (Reference number: 2019/139). No statistical test was used to determine adequate sample size and the study did not use blinding.

PCIP-seq

Total genomic DNA isolation was carried out using the Qiagen AllPrep DNA/RNA/miRNA kit (BLV, HTLV-1 and HPV infected individuals) or the Qiagen DNeasy Blood & Tissue Kit (HIV-1 patients) according to manufacturer's protocol. High molecular weight DNA was sheared to ~8kb using Covaris g-tubesTM (Woburn, MA) or a Megaruptor (Diagenode), followed by end-repair using the NEBNext EndRepair Module (New England Biolabs). Intramolecular circularization was achieved by overnight incubation at 16°C with T4 DNA Ligase. Remaining linear DNA was removed with Plasmid-Safe-ATP-Dependent DNAse (Epicentre, Madison WI). Guide RNAs were designed using chopchop (http://chopchop.cbu.uib.no/index.php). The EnGen™ sgRNA Template Oligo Designer (http://nebiocalculator.neb.com/#!/sgrna) provided the final oligo sequence. Oligos were synthesized by Integrated DNA Technologies (IDT). Oligos were pooled and guide RNAs synthesized with the EnGen sgRNA Synthesis kit, S. pyogenes (New England Biolabs). Selective linearization reactions were performed with the Cas-9 nuclease, S. pyogenes (New England Biolabs). (See Example 3 for the rationale behind using of CRISPR-cas9 to cleave the circular DNA). PCR primers flanking the cut sites were designed using primer3 (http://bioinfo.ut.ee/primer3/). Primers were tailed to facilitate the addition of Oxford Nanopore indexes in a subsequent PCR reaction. The linearized fragments were PCR amplified with LongAmp Taq DNA Polymerase (New England Biolabs) and purified using 1x AmpureXP beads, (Beckman Coulter). A second PCR added the appropriate Oxford Nanopore index. PCR products were visualized on a 1% agarose gel, purified using 1x AmpureXP beads and quantified on a Nanodrop spectrophotometer. Indexed PCR products

were multiplexed and Oxford Nanopore libraries prepared with either the Ligation Sequencing Kit 1D (SQK-LSK108) or 1D^2 Sequencing Kit (SQK-LSK308) (only the 1D were used) The resulting libraries were sequenced on Oxford Nanopore MinION R9.4 or R9.5 flow cells respectively. The endogenous retrovirus libraries were base called using albacore 2.3.1, all other PCIP-seq libraries were base called with Guppy 3.1.5 (https://nanoporetech.com) using the "high accuracy" base calling model. For the endogenous retrovirus libraries, demultiplexing was carried out via porechop (https://github.com/rrwick/Porechop) using the default setting. The HIV, HTLV-1, BLV and HPV PCIP-seq libraries were subjected to a more stringent demultiplexing with the guppy_barcoder (https://nanoporetech.com) tool using the --require_barcodes_both_ends option. The output was also passed through porechop, again barcodes were required on both ends, adapter sequence was trimmed and reads with middle adapters were discarded. Oligos used can be found in Tables 1 and 2.

Table 1: Guide RNA oligo's.

Guide Pool	Guide RNA Oligos	SEQ ID NO:
BLV-Pool-A (used in Bov & OAR)		
2563-BLV-Guide31_5PA	TTCTAATACGACTCACTATAGTCTGAGGGGAGATACCAGCGTTTTAGAGCTAGA	7
2564-BLV-Guide32_5PA	TTCTAATACGACTCACTATAGAAGACCCAAAACGCCGCCGAGTTTTAGAGCTAGA	8
2565-BLV-Guide33_5PA	TTCTAATACGACTCACTATAGCACCCCCTCGGCGGCGTTTTGTTTTAGAGCTAGA	9
2597-BLV-Guide43_3PA	TTCTAATACGACTCACTATAGACAGCCGGAGGGGGTCCACAGTTTTAGAGCTAGA	10
2598-BLV-Guide44_3PA	TTCTAATACGACTCACTATAGTTAGTAACGCATCCTGTCCTGTTTTAGAGCTAGA	11
2599-BLV-Guide45_3PA	TTCTAATACGACTCACTATAGCCCTCCTTGTGGACCCCCTCGTTTTAGAGCTAGA	12
2560-BLV-Guide46_3PA	TTCTAATACGACTCACTATAGCAAAGACGGACAGCCGGAGGGTTTTAGAGCTAGA	13
BLV Pool B (used in OAR)		
2570-BLV-Guide34_5PB	TTCTAATACGACTCACTATAGCTTCTGGGGCCGATGCACCCGTTTTAGAGCTAGA	14
2571-BLV-Guide35_5PB	TTCTAATACGACTCACTATAGCGAAGTGCTCTCAAACGATGGTTTTAGAGCTAGA	15
2572-BLV-Guide36_5PB	TTCTAATACGACTCACTATAGAACGGCGGGGGGGTCATAAGGTTTTAGAGCTAGA	16
2584-BLV-Guide40_3PB	TTCTAATACGACTCACTATAGGTTAGGAATAGGTCGATCGGTTTTAGAGCTAGA	17
2585-BLV-Guide41_3PB	TTCTAATACGACTCACTATAGTAACCGGTCGCATGGGGAAGGTTTTAGAGCTAGA	18
2586-BLV-Guide42_3PB	TTCTAATACGACTCACTATAGAGGAAGCGTTGTAAGGCCTGGTTTTAGAGCTAGA	19
BLV BOV Pool B (used in OAR)		

2570-BLV-Guide34_5PB	TTCTAATACGACTCACTATAGCTTCTGGGGCCGATGCACCCGTTTTAGAGCTAGA	20
2571-BLV-Guide35_5PB	TTCTAATACGACTCACTATAGCGAAGTGCTCTCAAACGATGGTTTTAGAGCTAGA	21
2572-BLV-Guide36_5PB	TTCTAATACGACTCACTATAGAACGGCGGGGGGGTCATAAGGTTTTAGAGCTAGA	22
2584-BLV-Guide40_3PB	TTCTAATACGACTCACTATAGGTTAGGAATAGGTCGATCGGTTTTAGAGCTAGA	23
2585-BLV-Guide41_3PB	TTCTAATACGACTCACTATAGTAACCGGTCGCATGGGGAAGGTTTTAGAGCTAGA	24
2691-BLV-Guide48_3PB	TTCTAATACGACTCACTATAGCTGCCCCTTATCCAAACGCCGTTTTAGAGCTAGA	25
BosT ERV Pool A		
2652-BosT_ERV_G7-PB5	TTCTAATACGACTCACTATAGAGGTTGTTCCTGAGTAGTCAGTTTTAGAGCTAGA	26
2663-BosT_ERV_G8-PB5	TTCTAATACGACTCACTATAGTGTTCCTCATCCCTATCTTTGTTTTAGAGCTAGA	27
2664-BosT_ERV_G9-PB5	TTCTAATACGACTCACTATAGACAACTAAATATCACTCTGAGTTTTAGAGCTAGA	28
BosT ERV Pool B		
2657-BosT_ERV_G10-PC3	TTCTAATACGACTCACTATAGCAAGGTAGCGTAGCCGAGGAGTTTTAGAGCTAGA	29
2658-BosT_ERV_G11-PC3	TTCTAATACGACTCACTATAGAAATCATTTGCTGTTCCAGGTTTTAGAGCTAGA	30
2659-BosT_ERV_G11-PC3	TTCTAATACGACTCACTATAGGGGTGTTACACATATCCACGTTTTAGAGCTAGA	31
Oar JSRV Pool A		
2627-JSRV_G9-5PA	TTCTAATACGACTCACTATAGTCGAGACCAGCCACAACAGAGTTTTAGAGCTAGA	32
2628-JSRV_G10-5PA	TTCTAATACGACTCACTATAGGGTTGCTTTCAACCCCCTCGTTTTAGAGCTAGA	33
2629-JSRV_G11-5PA	TTCTAATACGACTCACTATAGACTATTGCTTTACAGAACGCGTTTTAGAGCTAGA	34
2642-JSRV_G18-3PA	TTCTAATACGACTCACTATAGTTACAGCGGATACAAAACGGTTTTAGAGCTAGA	35
2643-JSRV_G19-3PA	TTCTAATACGACTCACTATAGAAGGCTGGTACGCGCGGCAGGTTTTAGAGCTAGA	36
2644-JSRV_G20-3PA	TTCTAATACGACTCACTATAGATGTCGAGCACGAATTGCATGTTTTAGAGCTAGA	37
Oar JSRV Pool B		
2632-JSRV_G12-5PB	TTCTAATACGACTCACTATAGATCTTTCAAAAGTCCGGCAGTTTTAGAGCTAGA	38
2633-JSRV_G13-5PB	TTCTAATACGACTCACTATAGCTGATGTTAACCGACAGCAGTTTTAGAGCTAGA	39
2634-JSRV_G14-5PB	TTCTAATACGACTCACTATAGCACAAATATCAAATGCGGCTGTTTTAGAGCTAGA	40
2637-JSRV_G15-3PB	TTCTAATACGACTCACTATAGGCTCAGACCTCTTTTAGGAGTTTTAGAGCTAGA	41
2638-JSRV_G16-3PB	TTCTAATACGACTCACTATAGTTCTGACTTTCCGTGGGATAGTTTTAGAGCTAGA	42
2639-JSRV_G17-3PB	TTCTAATACGACTCACTATAGATTTTGTAAAAAATTATCGAGTTTTAGAGCTAGA	43
HTLV1 Pool A		
2604-HTLV1_G21-5PA	TTCTAATACGACTCACTATAGCTGGTGGAAATCGTAACTGGGTTTTAGAGCTAGA	44
2605-HTLV1_G22-5PA	TTCTAATACGACTCACTATAGTCCCAAAAGGATACCCCGGCGTTTTAGAGCTAGA	45
2606-HTLV1_G23-5PA	TTCTAATACGACTCACTATAGTAAAATTTCATTCACCCGGCGTTTTAGAGCTAGA	46
2611-HTLV1_G24-3PA	TTCTAATACGACTCACTATAGCGGGGTGGCAAAAAATCACGGTTTTAGAGCTAGA	47

2612-HTLV1_G25-3PA	TTCTAATACGACTCACTATAGGGTGTACAGGTTTTGGGGCGTTTTAGAGCTAGA	48
2613-HTLV1_G26-3PA	TTCTAATACGACTCACTATAGTTTGCCACCCCGGCCAGCTCGTTTTAGAGCTAGA	49
HTLV1 Pool B		
2616-HTLV1_G27-5PB	TTCTAATACGACTCACTATAGCATGACTGGAAGGACTTGGGGTTTTAGAGCTAGA	50
2617-HTLV1_G28-5PB	TTCTAATACGACTCACTATAGGATGGTCTGCATAAACTGGGTTTTAGAGCTAGA	51
2618-HTLV1_G29-5PB	TTCTAATACGACTCACTATAGCAAACTGCTGCACCGCAAGCGTTTTAGAGCTAGA	52
2619-HTLV1_G30-3PB	TTCTAATACGACTCACTATAGGAAATCATAGGCGTGCCATGTTTTAGAGCTAGA	53
2620-HTLV1_G31-3PB	TTCTAATACGACTCACTATAGGCTGGCCATCTTTAGGGCAGTTTTAGAGCTAGA	54
2621-HTLV1_G32-3PB	TTCTAATACGACTCACTATAGAGGACTGTAGTACTAAAGAGTTTTAGAGCTAGA	55
2622-HTLV1_G33-3PB	TTCTAATACGACTCACTATAGATGGCACGCCTATGATTTCCGTTTTAGAGCTAGA	56
HIV U1 Pool A		
2667-HIV_G1-5PA	TTCTAATACGACTCACTATAGAGAGCGTCGGTATTAAGCGGGTTTTAGAGCTAGA	57
2668-HIV_G2-5PA	TTCTAATACGACTCACTATAGCGGGGGAGAATTAGATAAAGTTTTAGAGCTAGA	58
2681-HIV_G9-3PA	TTCTAATACGACTCACTATAGAGGCGGGTCTGGAACGATAAGTTTTAGAGCTAGA	59
2682-HIV_G10-3PA	TTCTAATACGACTCACTATAGCACTCATCTGGGTCGATCTGGTTTTAGAGCTAGA	60
2683-HIV_G11-3PA	TTCTAATACGACTCACTATAGAATCCATTCACTAATGGTCGTTTTAGAGCTAGA	61
HIV U1 Pool B		
2671-HIV_G3-5PB	TTCTAATACGACTCACTATAGCATGCAGGGCCTATTGCACCGTTTTAGAGCTAGA	62
2672-HIV_G4-5PB	TTCTAATACGACTCACTATAGATTGCATCCAGTGCATGCAGTTTTAGAGCTAGA	63
2673-HIV_G5-5PB	TTCTAATACGACTCACTATAGCAATAGGCCCTGCATGCACGTTTTAGAGCTAGA	64
2676-HIV_G6-3PB	TTCTAATACGACTCACTATAGCAAAACGTAGTATGAGTGGAGTTTTAGAGCTAGA	65
2677-HIV_G7-3PB	TTCTAATACGACTCACTATAGCTACTAATGCTAATTGTGCCGTTTTAGAGCTAGA	66
2678-HIV_G8-3PB	TTCTAATACGACTCACTATAGCGAACTGAACCAGCAGCAGAGTTTTAGAGCTAGA	67
HPV18 Region 1 Guide RNA		
1_HPV18_R1_guide1	TTCTAATACGACTCACTATAGTGCTGCAACCGAGCACGACGTTTTAGAGCTAGA	68
2_HPV18_R1_guide2	TTCTAATACGACTCACTATAGTGCTCGGTTGCAGCACGAAGTTTTAGAGCTAGA	69
3_HPV18_R1_guide3	TTCTAATACGACTCACTATAGCGACGATTTCACAACATAGCGTTTTAGAGCTAGA	70
HPV18 Region 2 Guide RNA		
8_HPV18_R2_guide4	TTCTAATACGACTCACTATAGATTTTAGAGGATTGGAACTTGTTTTAGAGCTAGA	71
9_HPV18_R2_guide5	TTCTAATACGACTCACTATAGTCTGCTATACTGCTTAAATTGTTTTAGAGCTAGA	72
10_HPV18_R2_guide6	TTCTAATACGACTCACTATAGCATCATATTGCCCAGGTACGTTTTAGAGCTAGA	73
HPV16_E6-E7 Guide RNA		

3261_HPV16_E6-E7_G1	TTCTAATACGACTCACTATAGCTAATTAACAAATCACACAAGTTTTAGAGCTAGA	74
3262_HPV16_E6-E7_G2	TTCTAATACGACTCACTATAGATTCCATAATATAAGGGGTGTTTTAGAGCTAGA	75
3263_HPV16_E6-E7_G3	TTCTAATACGACTCACTATAGCAACAAGACATACATCGACGTTTTAGAGCTAGA	76
HPV16_L1		
3266_HPV16_L1_G1	TTCTAATACGACTCACTATAGCCACCTATAGGGGAACACTGGTTTTAGAGCTAGA	77
3267_HPV16_L1_G2	TTCTAATACGACTCACTATAGACCTACCTCAACACCTACACGTTTTAGAGCTAGA	78
3268_HPV16_L1_G3	TTCTAATACGACTCACTATAGTAATAGAGAATGTATATCTAGTTTTAGAGCTAGA	79

Table 2: Primers used for amplification of linearized DNA molecules

	PCR primers	SEQ ID NO:	
BLV Pool A			
2568-BLV_5PA-minION-F	TTTCTGTTGGTGCTGATATTGCGCGACCCTCTCCTAGCGATTTT	80	psp344:718-739
2595-BLV_5PA-minION-R	ACTTGCCTGTCGCTCTATCTTCGTTAGGGTTCCGGGGTGATCAA	81	psp344:551-572
2601-BLV_3PA-minION-F	TTTCTGTTGGTGCTGATATTGCCTCCACCCTTTTGACGCTATGC	82	psp344:7815-7836
2602-BLV_3PA-minION-R	ACTTGCCTGTCGCTCTATCTTCATTGGCATTGGTAGGGCTGGAA	83	psp344:7585-7606
BLV Pool B			
2575-BLV_5PB-minION-F	TTTCTGTTGGTGCTGATATTGCCCCGCCGTTTTGCCAATCATAT	84	psp344:944-965
2576-BLV_5PB-minION-R	ACTTGCCTGTCGCTCTATCTTCTTTAGGGTGGCCAAGACAAGGG	85	psp344:849-870
2589-BLV_3PB-minION-F	TTTCTGTTGGTGCTGATATTGCTCAGAATTGGTTGCTAGCGGGA	86	psp344:8089-8110
2603-BLV_3PB-minION-R	ACTTGCCTGTCGCTCTATCTTCTTTGGATAAGGGGCAGCTCGAA	87	psp344:7933-7954
BLV BOV Pool B			
2575-BLV_5PB-minION-F	TTTCTGTTGGTGCTGATATTGCCCCGCCGTTTTGCCAATCATAT	88	psp344:944-965
2576-BLV_5PB-minION-R	ACTTGCCTGTCGCTCTATCTTCTTTAGGGTGGCCAAGACAAGGG	89	psp344:849-870
2690-BLV_3PB-minION-F	TTTCTGTTGGTGCTGATATTGCGGTCCAGTCCTCAGGCCTTAC	90	psp344:8036-8056
2603-BLV_3PB-minION-R	ACTTGCCTGTCGCTCTATCTTCTTTGGATAAGGGGCAGCTCGAA	91	psp344:7933-7954
BosT ERV Pool A			
2650-BosT_ERV_PB5-F	TTTCTGTTGGTGCTGATATTGCCTGTCAGACCATCCGCTCCTAG	92	ChrX_ERV_denovo:2305-2326
2651-BosT_ERV_PB5-R	ACTTGCCTGTCGCTCTATCTTCTAGTCAGGCGGGTCTTCGTTTT	93	ChrX_ERV_denovo:2095-2116
BosT ERV Pool B			
2655-BosT_ERV_PC3-F	TTTCTGTTGGTGCTGATATTGCTCTTCGGCAGAGCATTCAGAGG	94	ChrX_ERV_denovo:5718-5739
2656-BosT_ERV_PC3-R	ACTTGCCTGTCGCTCTATCTTCAAGTAAGCCCACAAACCGTCGT	95	ChrX_ERV_denovo:5133-5154
Oar JSRV Pool A			
2625-JSRV-5PA-F	TTTCTGTTGGTGCTGATATTGCCCTCCACCGTCTGAGAACATGT	96	enJSRV-7:1269-1290

2626-JSRV-5PA-R	ACTTGCCTGTCGCTCTATCTTCAGCATACCTGGGTTCCGAATCA	97	enJSRV-7:920-941
2640-JSRV-3PA-F	TTTCTGTTGGTGCTGATATTGCGAACCGGACCTCTCGACATTCC	98	enJSRV-7:6216-6237
2641-JSRV-3PA-R	ACTTGCCTGTCGCTCTATCTTCAAACACAAACATGCCCTCGTCC	99	enJSRV-7:5650-5671
Oar JSRV Pool B			
2630-JSRV-5PB-F	TTTCTGTTGGTGCTGATATTGCGGGACCTGATGAGCCTTACCAG	100	enJSRV-7:1796-1817
2631-JSRV-5PB-R	ACTTGCCTGTCGCTCTATCTTCGCAATGGTGAATGGAGCGGTAG	101	enJSRV-7:1453-1474
2635-JSRV-3PB-F	TTTCTGTTGGTGCTGATATTGCCCTTCATTCACTGTGGCGAAGT	102	enJSRV-7:7306-7327
2636-JSRV-3PB-R	ACTTGCCTGTCGCTCTATCTTCGTAAGGAACACAAGCTCGGGGA	103	enJSRV-7:6553-6574
HTLV1 Pool A			
2607-HTLV1-5PA-F	TTTCTGTTGGTGCTGATATTGCTCATCCAAACCCAAGCCCAGAT	104	HTLV_ATK:1083-1104
2608-HTLV1-5PA-R	ACTTGCCTGTCGCTCTATCTTCGGACCGGGTTCTAGGCGATATG	105	HTLV_ATK:915-936
2609-HTLV1-3PA-F	TTTCTGTTGGTGCTGATATTGCTCTACCCGAAGACTGTTTGCCC	106	HTLV_ATK:7941-7962
2610-HTLV1-3PA-R	ACTTGCCTGTCGCTCTATCTTCTTGTATGAGTGATTGGCGGGGT	107	HTLV_ATK:7591-7612
HTLV1 Pool B			
2614-HTLV1-5PB-F	TTTCTGTTGGTGCTGATATTGCAAAGACCTCCAAGACCTCCTGC	108	HTLV_ATK:1370-1391
2615-HTLV1-5PB-R	ACTTGCCTGTCGCTCTATCTTCCGTAGGCTCAACATAGGGAGGG	109	HTLV_ATK:1177-1198
2623-HTLV1-3PB-F	TTTCTGTTGGTGCTGATATTGCCTCTCACACGGCCTCATACAGT	110	HTLV_ATK:8194-8215
2624-HTLV1-3PB-R	ACTTGCCTGTCGCTCTATCTTCGAGTGGTGAGGGTTGAGTGGAA	111	HTLV_ATK:8029-8050
HIV U1 Pool A			
2665-HIV-5PA-F	TTTCTGTTGGTGCTGATATTGCAaaattcggttaaggccagggg	112	HIV_U1:841-862
2666-HIV-5PA-R	ACTTGCCTGTCGCTCTATCTTCCtcgcacccatctctctccttc	113	HIV_U1:779-800
2679-HIV-3PA-F	TTTCTGTTGGTGCTGATATTGCGctaccaccgcttgagagactt	114	HIV_U1:8461-8482
2680-HIV-3PA-R	ACTTGCCTGTCGCTCTATCTTCaccaattccacaaacttgccca	115	HIV_U1:8157-8178
HIV U1 Pool B			
2669-HIV-5PB-F	TTTCTGTTGGTGCTGATATTGCCcaggccagatgagagaaccaa	116	HIV_U1:1462-1483
2670-HIV-5PB-R	ACTTGCCTGTCGCTCTATCTTCtcccattctgcagcttcctcat	117	HIV_U1:1406-1427
2674-HIV-3PB-F	TTTCTGTTGGTGCTGATATTGCgaggaggaggaggtgggttttc	118	HIV_U1:8917-8938
2675-HIV-3PB-R	ACTTGCCTGTCGCTCTATCTTCtgaccacttgccaccatctta	119	HIV_U1:8730-8751
HPV18 Pool A			
4_HPV18_R1_Left	ctccaacgacgcagagaaacac	120	
5_HPV18_R1_Right	ggattcaacggtttctggcacc	121	
HPV18 Pool B			
11_HPV18_R2_Left	ttttggttcaggctggattgcg	122	
12_HPV18_R2_Right	agaatacacacagctgccaggt	123	
HPV16_E6-E7			

3259_HPV16_E6-E7	AACCGGACAGAGCCCATTACAA	124	
3260_HPV16_E6-E7	AGTCATATACCTCACGTCGCAGT	125	
HPV16_L1			
3264_HPV16_L1	ACTGGCTTTGGTGCTATGGACT	126	
3265_HPV16_L1	CAAACCAGCCGCTGTGTATCTG	127	

Identification of proviral integration sites in PCIP-seq

Reads were mapped with Minimap2[55] to the host genome with the proviral genome as a separate chromosome. In-house R-scripts were used to identify integration sites (IS). Briefly, chimeric reads that partially mapped to at least one extremity of the proviral genome were used to extract virus-host junctions and shear sites. Junctions within a 200bp window were clustered together to form an "IS cluster", compensating for sequencing/mapping errors. The IS retained corresponded to the position supported by the highest number of virus-host junctions in each IS cluster. Clone abundance was estimated based on the number of reads supporting each IS cluster. Reads sharing the same integration site and same shear site were considered PCR duplicates. Custom software, code description and detailed outline of the workflow are available on Github: https://github.com/GIGA-AnimalGenomics-BLV/PCIP.

Measure of proviral load (PVL) and identification of proviral integration sites (Illumina)

PVLs and integration sites of HTLV-1- and BLV-positive individuals were determined as previously described in Rosewick et al 2017[7] and Artesi et al 2017[20]. PVL represents the percentage of infected cells, considering a single proviral integration per cell. Total HIV-1 DNA content of CD4 T-cell DNA isolates was measured by digital droplet PCR (ddPCR, QX200 platform, Bio-Rad, Temse, Belgium), as described by Rutsaert et al.[56] The DNA was subjected to a restriction digest with EcoRI (Promega, Leiden, The Netherlands) for one hour, and diluted 1:2 in nuclease free water. HIV-1 DNA was measured in triplicate using 4 µL of the diluted DNA as input into a 20µL reaction, while the RPP30 reference gene was measured in duplicate using 1 µL as input. Primers and probes are summarized in Table 3. Thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 56°C for 60 s, followed by 98°C for 10 min. Data was analyzed with the ddpcRquant analysis software[57].

Table 3

Assay	*Location*	*Primer*	*Label*	*Temp. (°C)*	*Sequence*
Total HIV-1 DNA	HIV LTR	Forward			5'-GCCTCAATAAAGCTTGCC-3' (SEQ ID NO:128)
	HIV LTR-Gag inter	Reverse			5'-GGCGCCACTGCTAGAGATTTT-3' (SEQ ID NO:129)
	HIV LTR	Probe	MGB/FAM	56	5'-AAGTRGTGTGTGCCC-3' (SEQ ID NO:130)
RPP30	human RPP30 gene	Forward			5'-AGATTTGGACCTGCGAGCG-3' (SEQ ID NO:131)
	human RPP30 gene	Reverse			5'-GAGCGGCTGTCTCCACAAGT-3' (SEQ ID NO:132)
	human RPP30 gene	Probe	HEX	56	5'-TTCTGACCTGAAGGCTCTGCGCG-3' (SEQ ID NO:133)

Variant Calling

After PCR duplicate removal, proviruses with an IS supported by more than 10 reads were retained for further processing. SNPs were identified using LoFreq[22] with default parameters, only SNPs with an allele frequency of >0.6 in the provirus associated with the insertion site were considered. We also called variants on proviruses supported by more than 10 reads without PCR duplicate removal (this greatly increased the number of proviruses examined). This data was used to explore the number of proviruses carrying the Tax 303 variant. Deletions were called on proviruses supported by more than 10 reads without PCR duplicate removal using an in house R-scripts. Briefly, samtools pileup[58] was used to calculate/compute coverage and deletions at base resolution. We used the changepoint detection algorithm PELT[59] to identify genomic windows showing an abrupt change in coverage. Windows that showed at least a 4-fold increase in the frequency of deletions (absence of a nucleotide for that position within a read) were flagged as deletions and visually confirmed in IGV[60].

HIV-1 proviral sequences

Sequences of the two major proviruses integrated in chr2 (SEQ ID NO:5) and chrX (SEQ ID NO:4) of the U1 cell line were generated by initially mapping the reads from both platforms to the HIV-1 provirus, isolate NY5 (GenBank: M38431.1), where the 5'LTR sequence is appended to the end of the sequence to produce a full-length HIV-1 proviral genome reference. The sequence was then manually curated to produce the sequence for each provirus. To check for recombination, reads of selected clones were mapped to the sequence from the chrX provirus and the patterns of SNPs examined to determine if the variants matched the chrX or chr2 proviruses.

Endogenous retroviruses

The sequence of bovine *APOB* ERV (SEQ ID NO:6) was generated by PCR amplifying the full length ERV with LongAmp Taq DNA Polymerase (New England Biolabs) from a Holstein suffering from cholesterol deficiency. The resultant PCR product was sequenced on the Illumina platform as described below. It was also sequenced with an Oxford Nanopore MinION R7 flow cell as previously described[29]. Full length sequence of the element was generated via manual curation. Guide RNAs and primer pairs were designed using this ERV reference. For the Ovine ERV we used the previously published enJSRV-7 sequence[40] as a reference to design PCIP-seq guide RNAs and PCR primers.

As the ovine and bovine genome contains sequences matching the ERV, mapping ERV PCIP-seq reads back to the reference genome creates a large pileup of reads in these regions. To avoid this, prior to mapping to the reference we first used BLAST[61] to identify the regions in the reference genome containing sequences matching the ERV, we then used BEDtools[62] to mask those regions. The appropriate ERV reference was then added as an additional chromosome in the reference.

PCR validation and Illumina sequencing

Clone specific PCR products were generated by placing primers in the flanking DNA as well as inside the provirus. LongAmp Taq DNA Polymerase (New England Biolabs) was used for amplification following the manufacturers guidelines. Resultant PCR products were sheared to ~400bp using the Bioruptor Pico (Diagenode) and Nextera XT indexes added as previously described[29]. Illumina PCIP-seq libraries were generated in the same manner. Sequencing was carried out on either an Illumina MiSeq or NextSeq 500. Clone specific PCR products sequenced on Nanopore were indexed by PCR, multiplexed and libraries prepared using the Ligation Sequencing Kit 1D (SQK-LSK108) and sequenced on a MinION R9.4 flow cell. Oligos used can be found in Tables 4-7.

Table 4: Primers used for clone specific validation of SNPs

Ovine 220_122013

Provirus	POS in BLV genome	REF	ALT	BLV Oligo	Oligo location in BLV Provirus	Host Oligo	Location in Host
OAR12_62009791_62009791	7925	T	G	TTTCAGAGGGCGGAGAAACA (SEQ ID NO: 134)	4648-4667	CACCCTGAGCCTCCATACAT (SEQ ID NO:137)	chr12:62010099-62010118
OAR2_248506820_248507220	466	T	C	TTTAGCAAACGCCAGGGAAC (SEQ ID NO:135)	4797-4816	GCGAATCTCTGTCTTGCTGG (SEQ ID NO:138)	chr2:248506994-248507013
OAR5_60508711_60508719	7511	G	A	TTTCAGAGGGCGGAGAAACA (SEQ ID NO:136)	4648-4667	AACTCTATGGCTGGAAGGACA (SEQ ID NO:139)	chr5:60509280-60509300

Ovine 221_022016 & 221_032014

Provirus	POS in BLV genome	REF	ALT	BLV Oligo	Oligo locaion in BLV Provirus	Host Oligo	Location
OARX_115780553_115780560	6251	G	A	TTTCAGAGGGCGGAGAAACA (SEQ ID NO: 140)	4648-4667	AGGTGGAGATGATGTGTGCA (SEQ ID NO: 146)	chrX:115781164-115781183
OAR3_68849355_68850177	973	G	A	TTTAGCAAACGCCAGGGAAC (SEQ ID NO: 141)	4797-4816	ACCTCACACCAAAACGAAGC (SEQ ID NO: 147)	chr3:68849738-68849757
"	2917	G	A	"	"	"	"
"	3139	C	T	"	"	"	"
OAR8_80138768_80138775	3407	T	C	TTTAGCAAACGCCAGGGAAC (SEQ ID NO: 142)	4797-4816	GTGACTTGTTTGCCTCCCTG (SEQ ID NO: 148)	chr8:80137900-80137919
OAR2_56698159_56698164	7524	C	A	TTTAGCAAACGCCAGGGAAC (SEQ ID NO: 143)	4797-4816	TTCATGTGCTTCCGTGGTTG (SEQ ID NO: 149)	chr2:56698504-56698523

OAR7_72660067_72660073	7191	G	A	TTTCAGAGGGCGGAGAAACA (SEQ ID NO: 144)	4648-4667	AGAGGCCTGAGTGTTTTGGT (SEQ ID NO: 150)	chr7:72660692-72660711
OAR8_80151001_80151007	5305	G	A	TTTCAGAGGGCGGAGAAACA (SEQ ID NO: 145)	4648-4667	GACCCACATCAGTTGCCTTC (SEQ ID NO: 151)	chr8:80151348-80151367

Bovine 14[3]9

Provirus	POS in BLV genome	REF	ALT	BLV Oligo	Oligo locaion in BLV Provirus	Host Oligo	Location
24_41573470_41573476	3415	A	G	GGGGCTCGCAATCATATGTG (SEQ ID NO: 152)	5143-5162	CTTGAACTCCGGGACCTTCT (SEQ ID NO: 166)	chr24:41574183-41574202
22_48070162_48070168	3470	T	G	GGGGCTCGCAATCATATGTG (SEQ ID NO: 153)	5143-5162	TCGAAAAGGCCAAGTACCCT (SEQ ID NO: 167)	chr22:48070630-48070649
18_57045658_57045664	3440	T	C	GGGGCTCGCAATCATATGTG (SEQ ID NO: 154)	5143-5162	GATGGGATGAGGTCAGGAGG (SEQ ID NO: 168)	chr18:57045372-57045391
18_61039250_61039250	453	T	C	GGGGCTCGCAATCATATGTG (SEQ ID NO: 155)	5143-5162	ACAGGCAGGATCTTTGTGGA (SEQ ID NO: 169)	chr18:61039161-61039180
2_5529599_5529704	106	C	T	GGGGCTCGCAATCATATGTG (SEQ ID NO: 156)	5143-5162	GCACACTGTCCTGAGAtcca (SEQ ID NO: 170)	chr2:5529276-5529295
"	8295	C	T	AGCCCTCTGGACTCACAATC (SEQ ID NO: 157)	4562-4581	CCAGTGCATGCttaatcgct (SEQ ID NO: 171)	chr2:5530006-5530025
2_54238495_54238502	93	T	C	GGGGCTCGCAATCATATGTG (SEQ ID NO: 158)	5143-5162	AATCCGTTCATGGTTCCGTG (SEQ ID NO: 172)	chr2:54238966-54238985
"	7437	T	C	AGCCCTCTGGACTCACAATC	4562-4581	GCTGCTAATTTGACTGGCCA	chr2:54237331-54237350

				(SEQ ID NO: 159)		(SEQ ID NO: 173)	
"	8282	T	C	"	"	"	"
21_45410573_45410985	2885	C	A	GGGGCTCGCAATCATA TGTG (SEQ ID NO: 160)	5143-5162	CTCGGGGAGACAGAAA ACCT (SEQ ID NO: 174)	chr21:45410493-45410512
29_41063804_41063804	3662	A	G	AGCCCTCTGGACTCACA ATC (SEQ ID NO: 161)	4562-4581	CTTCCCTGCTCCATCCCT AG (SEQ ID NO: 175)	chr29:41062629-41062648
"	8642	T	C	GGGGCTCGCAATCATA TGTG (SEQ ID NO: 162)	5143-5162	CAGCTTACTCCACCCTTC CA (SEQ ID NO: 176)	chr29:41064575-41064594
3_87619443_87619450	453	T	C	AGCCCTCTGGACTCACA ATC (SEQ ID NO: 163)	4562-4581	GCAAGAGAAGAGAGTG GGGT (SEQ ID NO: 177)	chr3:87618300-87618319
"	8642	T	C	GGGGCTCGCAATCATA TGTG (SEQ ID NO: 164)	5143-5162	TCTAATCCCCAAGCTGT GCA (SEQ ID NO: 178)	chr3:87619588-87619607
1_150385145_150385351	5859	G	A	AGCCCTCTGGACTCACA ATC (SEQ ID NO: 165)	4562-4581	CGACAAGCCTGGTAAG ATGC (SEQ ID NO: 179)	chr1:150385624-150385643

Table 5: Primers for clone specific validation of SV

Bovine 1439

Provirus	Aprox start BLV	Aprox end BLV	type	BLV Oligo	Oligo locaion in BLV Provirus	Host Oligo	Location in Host
1_150385145_150385351	3451	3474	DEL	GGGGCTCGCAATCATA TGTG (SEQ ID NO: 180)	5143-5162	GTGGGACGGTGTTTGA AGTC (SEQ ID NO: 188)	chr1:150384631-150384650
2_124084208_124084213	391	406	DEL	GAGGCATCGATAGCAT GGTCCT (SEQ ID NO: 181)	1663-1684	TTCCCCAAGACTTTCCC AGGTC (SEQ ID NO: 189)	chr2:124084230-124084251

23_39892380_39892560	2364	2560	DEL	AAATCTGGGGCCACAATTGCAG (SEQ ID NO: 182)	3504-3525	TCCAGTGGCCGTGTATTTGTCT (SEQ ID NO: 190)	chr23:39893192-39893213
27_36582809_36582809	1	852	DEL	CCACCCTATTGCTTCCCTGA (SEQ ID NO: 183)	3950-3969	TTCCCTTAGCAGTCAGGTGG (SEQ ID NO: 191)	chr27:36583265-36583284
27_36582809_36582809	4522	5636	DEL	GGCATGAGTAGCTCCAGAGT (SEQ ID NO: 184)	4258-4277	AGGCCTTCACTCTAACCGTT (SEQ ID NO: 192)	chr27:36581475-36581494
3_45576532_45576538	2316	2336	DEL	AAATCTGGGGCCACAATTGCAG (SEQ ID NO: 185)	3504-3525	TACTGCCCATCACCCCTTCATC (SEQ ID NO193)	chr3:45576400-45576421
4_100234239_100234246	8296	8370	INS	AGCCCTCTGGACTCACAATC (SEQ ID NO: 186)	4562-4581	ACAAAACAGTCAAACAGGGCT (SEQ ID NO: 194)	chr4:100234688-100234708
5_51456241_51456285	1	4152	DEL	AGCGAGGAGAGTGAGAGTGAGA (SEQ ID NO: 187)	4882-4903	CCCCTGCATAAAATGAGGCCTG (SEQ ID NO: 195)	chr5:51456399-51456420

Ovine 221

Provirus	Aprox start BLV	Aprox end BLV	type	BLV Oligo	Oligo locaion in BLV Provirus	Host Oligo	Location in Host
OAR25_25097056_25097063	2325	4303	DEL	AGATTTCAGGGAAGTGGGGAGC (SEQ ID NO: 196)	6236-6257	TGCCTTCTCCGTTCCCAATTCT (SEQ ID NO: 202)	chr25:25097010-25097031
OARX_78143793_78143801	3284	6602	DEL	TGGATGTGGCTGGAATGTCT (SEQ ID NO: 197)	7063-7082	CACCAGGGAAGTCTTGTTGC (SEQ ID NO: 203)	chrX:78144637-78144656
OARX_78143793_78143801	3284	6602	DEL	AATTACAGGCGGTCTTGGGA (SEQ ID NO: 198)	3025-3044	CAGCCTCAGAGTTCCTTCCA (SEQ ID NO: 204)	chrX:78143342-78143361

OAR1_250672128_250672136	7365	7389	DEL	AAATGCCCAAAGAACGACGGTC (SEQ ID NO: 199)	4824-4845	AGCCTTCACAAGTCACCTCTCC (SEQ ID NO: 205)	chr1:250672354-250672375
OAR2_242159705_242159712	7017	7232	INS	CGAATCTTCCCCATGCAGCTTC (SEQ ID NO: 200)	6775-6796	GATGCCCTGGAATGGTTTGGTG (SEQ ID NO: 206)	chr2:242159088-242159109
OAR8_80161637_80161982	6502	6561	DEL	AAATGCCCAAAGAACGACGGTC (SEQ ID NO: 201)	4824-4845	TCCAGAAGAGGCAAAGCAAGGA (SEQ ID NO: 207)	chr8:80163636-80163657

Ovine 223

Provirus	Aprox start BLV	Aprox end BLV	type	BLV Oligo	Position of oligo in BLV Provirus	Host Oligo	Location in Host
OAR10_34545991_34546003	5298	5330	DEL	AAATGCCCAAAGAACGACGGTC (SEQ ID NO: 208)	4824-4845	AAGTCGAGCAAGGCACCTATGT (SEQ ID NO: 210)	chr10:34547689-34547710
OAR10_49266255_49266262	6512	6586	DEL	AAATGCCCAAAGAACGACGGTC (SEQ ID NO: 209)	4824-4845	TGGTTGTGGGTCATCATCGTCT (SEQ ID NO: 211)	chr10:49266300-49266321

Table 6: Primers for long range PCR to validate ERVs in the Bovine

ERV	Forward Oligo	Location	Reverse Oligo	Location
BTA8_37.3	GGCTGCCCTTCACTGAGAGTAA (SEQ ID NO: 212)	chr8:37362441-37362462	TTTACCCTTGGAGTGTGGCCTT (SEQ ID NO: 215)	chr8:37362889-37362910
BTA21_18.6	TGGCTAAGTTCCACCACACTCT (SEQ ID NO: 213)	chr21:18639407-18639428	GGGTCCTCTGTCCTCTGTCTTC (SEQ ID NO: 216)	chr21:18639907-18639928
BTA27_14.1	GGAGCAAGGTAGAGGGGTGAAG (SEQ ID NO: 214)	chr27:14152640-14152661	AGAGGGAAATCACACCGAAGCA (SEQ ID NO: 217)	chr27:14153202-14153223

Table 7: Primers for long range PCR to validate ERVs in the Ovine

ERV	Forward Oligo	Location	Reverse Oligo	Location
OAR1_86.0	GTTGTTGCATCTTCCGGTCCTG (SEQ ID NO: 218)	chr1:85959032-85959053	GGAGCCTCAACGACTCTGCTAA (SEQ ID NO: 225)	chr1:85964651-85964672
OAR3_39.2	TAGCCCAGCAAGAGTCTCCCTA (SEQ ID NO: 219)	chr3:39184853-39184874	CCCCTTCATAGCCCACTGGAAA (SEQ ID NO: 226)	chr3:39196544-39196565
OAR4_77.4	TTGATGTGAAGAGCCTGTGAGC (SEQ ID NO: 220)	chr4:77421367-77421388	CCAGCAACTCAGACAAACCAGG (SEQ ID NO: 227)	chr4:77421696-77421717
OAR13_16.7	GGCTTCAAACACACCTCACCTC (SEQ ID NO: 221)	chr13:16720272-16720293	AATGTGTAGATGGAGGCTGGGC (SEQ ID NO: 228)	chr13:16721090-16721111
OAR4_40.4	GAGATGGCCGTGTGTGACAAAG (SEQ ID NO: 222)	chr4:40492573-40492594	GCTAACAAACGGGTGGCAAAGA (SEQ ID NO: 229)	chr4:40493498-40493519
OAR5_73.0	TGAAAGACTCACTGTGGCCCAA (SEQ ID NO: 223)	chr5:73012745-73012766	CTGGGGAAGCCAAGCAAAGATG (SEQ ID NO: 230)	chr5:73013599-73013620
OAR13_66.0	ACTCTCTCCCAACATTGCCCTC (SEQ ID NO: 224)	chr13:66026352-66026373	ATTCTGGTGGTCTCTGTGGCTC (SEQ ID NO: 231)	chr13:66027161-66027182

BLV references

The sequence (SEQ ID NO:1) of the pBLV344 provirus was generated via a combination of Sanger and Illumina based sequencing with manual curation of the sequence to produce a full length proviral sequence. The consensus BLV sequences for the bovine samples 1439 & 1053 (SEQ ID NO:3,2) were generated by first mapping the PCIP-seq Nanopore reads to the pBLV344 provirus. We then used Nanopolish[63] to create an improved consensus. PCIP-seq libraries sequenced on the Illumina and Nanopore platform were mapped to this improved consensus visualized in IGV and manually corrected.

Genome references used

Sheep=OAR3.1; Cattle=UMD3.1; Human=hg38; For HTLV-1 integration sites hg19 was used; HPV18=GenBank: AY262282.1; Sequences of the exogenous and endogenous proviruses can be found in SEQ ID NO:1-SEQ ID NO:6.

Data availability

Sequence data that support the findings of this study have been deposited in the European Nucleotide Archive (ENA) hosted by the European Bioinformatics Institute (EMBL-EBI) and are accessible through study accession number PRJEB34495. All other relevant data are available within the article and its Supplementary Information files or from the corresponding authors upon reasonable request.

Code availability

The code and a detailed outline of the PCIP-seq analysis workflow are publicly available on Github: https://github.com/GIGA-AnimalGenomics-BLV/PCIP

Example 2: Overview of PCIP-seq (Pooled CRISPR Inverse PCR-sequencing)

The genome size of the viruses targeted ranged from 6.8 to 9.7kb, therefore we chose to shear the DNA to ~8kb in length. In most cases this creates two fragments for each provirus, one containing the 5' end with host DNA upstream of the insertion site and the second with the 3' end and downstream host DNA. Depending on the shear site the amount of host and proviral DNA in each fragment will vary (Fig. 1a). To facilitate identification of the provirus insertion site via inverse PCR we carry out intramolecular ligation, followed by digestion of the remaining linear DNA. To selectively linearize the circular DNA containing proviral sequences (this helps increase PCR efficiency), regions adjacent to the 5' and 3' LTRs in the provirus are targeted for CRISPR mediated cleavage. We sought a balance between ensuring that the majority of the reads contained part of the flanking DNA (for clone identification) while also generating sufficient reads extending into the midpoint of the provirus. We found that using a pool of CRISPR guides for each region increased the efficiency and by multiplexing the guide pools and PCR primers for the 5' and 3' ends we could generate coverage for the majority of a clonally expanded provirus in a single reaction (Fig. 1b). The multiplexed pool of guides and primers leaves coverage gaps in the regions flanked by the primers. To address these coverage gaps we designed a second set of guides and primers. Following separate CRISPR cleavage and PCR amplification the products of these two sets of guides and primers were combined for sequencing (Fig. 1c). This approach ensured that the complete provirus was sequenced (Fig. 1d).

Pooled CRISPR Inverse PCR sequencing (PCIP-seq) leverages long reads on the Oxford Nanopore MinION platform to sequence the insertion site and its associated provirus. The technique was applied to natural infections produced by three exogenous retroviruses, HTLV-1, BLV and HIV-1 as well as endogenous retroviruses in both cattle and sheep. The high efficiency of the method facilitated the identification of tens of thousands of insertion sites in a single sample. Thousands of SNPs and dozens of structural variants within proviruses were observed. While initially developed for retroviruses the method has also been successfully extended to DNA extracted from HPV positive PAP smears, where it could assist in identifying viral integrations associated with clonal expansion. An overview of the applications tested herein is provided in Table 8.

Table 8: Number of insertion sites (IS) identified via PCIP-seq. Chimeric reads = reads containing host and viral DNA. Largest clone % = insertion site with highest number of reads in that sample. PVL = Proviral Load. (Percentage cells carrying a single copy of integrated provirus or number proviral copies per 100 cells).

Sample name	Virus	Host	PVL	Template µg	raw reads	Chimeric reads (%)	Pure Host / Pure Viral reads	Insertion sites	Largest clone (%)
ATL2	HTLV-1	HSA	nd	4	81,219	68.21	0.0037 / 31.8	160	49.5
ATL100	HTLV-1	HSA	106	4	4,838	64.14	9.16 / 26.7	13	89.624
233	BLV	OAR	78.3	7	524,698	53.4	0.04 / 46.53	5311	5.22
221 (022016)	BLV	OAR	63	4	180,276	67.14	3.59 / 29.27	8023	0.625
221 (032014)	BLV	OAR	16	4	32,266	68.69	0.11 / 31.20	5374	0.279
220	BLV	OAR	3.8	2	44,876	67.38	0 / 32.62	1352	3.55
1439	BLV	BosT	45	3	181,055	70.52	0.19 / 29.29	5773	1.17
560	BLV	BosT	0.644	1	6,802	69.83	1.12 / 29.06	172	4.59
1053	BLV	BosT	23.5	6	367,454	72.13	0.04 / 27.83	17903	0.353
HIV_U1	HIV-1	HSA	200	2	94,086	54.66	2.75 / 42.59	728	47.2

Sample name	Virus	Host	PVL	Template µg	raw reads	Chimeric reads (%)	Pure Host / Pure Viral reads	Insertion sites	Largest clone (%)
Jurkat U1-0.1	HIV-1	HSA	0.2	5	252,913	43.33	0.04 / 56.62	4	71.7
Jurkat U1-0.01	HIV-1	HSA	0.02	5	234,421	43.33	0.04 / 56.52	2	90.2
Jurkat neg	HIV-1	HSA	0	5	12,137	0	100 / 0	0	0
HPV18_PX	HPV18	HAS	nd	4	180,550	21.36	0.29 / 78.35	55	nd
HPV18_PY	HPV18	HAS	nd	4	82,807	0.09	0.05 / 99.86	19	nd

Example 3: Rationale behind the use of CRISPR-cas9 to cleave circular DNA

It is established practice to linearize plasmids (generally via cutting with a restriction enzyme) prior to their use as template in PCR. It is believed that this avoids supercoiling and thereby increases PCR efficiency[67]. Following the same logic, we speculated that linearizing our circularized DNA could also increase PCR efficiency. Figure 8 shows an experiment carried out using 8µg of DNA from a BLV infected sheep with a proviral load of 82.6%. The DNA was circularized and linear DNA was eliminated (to prevent PCR amplification/recombination involving the remaining linear fragments) using plasmid safe DNase (see Example 1 for a complete description). One quarter of the resultant DNA was subject to CRISPR-cas9 cleavage using the Pool A guides, the second quarter was cleaved using the Pool B guides, the remaining half was kept aside. The linearized DNA was cleaned and used as template in 2x 50µl PCR reactions using the appropriate primer pairs for Pool A (PA) or Pool B (PB). For the uncut DNA half was used as template for 2x 50µl PCR reactions using the PA primers and the other half was used for 2x 50µl PCR reactions using the PB primers. Following 25 PCR cycles, 10µl of each reaction were loaded on a 1% agarose gel. As can be seen in Figure 8, the band intensity for the CRISPR-cas9 cut samples is higher. It should be noted that in lane 3 the PCR smear is shifted down, we generally discard these types of products as the fraction of host-virus fragments is low. (A=unshared genomic DNA, B=genomic DNA sheared to 8kb).

Following clean up and elution in ~40 µl of H_2O we took an equal volume (3µl) of each library and indexed them via PCR, in a 50 µl reaction volume and using 8 cycles. Again, following clean up, an equal volume of library was pooled and a nanopore library (LSK-

109) was prepared and sequenced on a r9.4 flow cell. Base calling and demultiplexing was carried out as described in Example 1. The results are outlined in Table 9. In addition the coverage of the resultant reads is shown in Figure 9.

Table 9

Lib	Treatment	DNA concentration PCR 1 (ng/ul)	DNA concentrationPCR 2 (ng/ul)	Raw reads	Chimeric reads %	Pure Host / Pure Viral reads (%)	Mean Length	N50	Median Length	Insertion sites PCIP	Largest clone PCIP (%)	Insertion sites Illumina	Largest clone Illumina (%)
1	PA-Cut BC31	22.52	69.48	113,485	55.6	0.25 / 44.2	2880.6	3855.0	2217.0	2122	25.8	1700	30.849
2	PA-Cut BC32	26.18	72.06	137,109	54.1	0.47 / 45.4	2770.6	3710.0	2141.0	2216	24.7	"	"
3	PB-Cut BC33	71.85	63.7	6,844	1.01	98.5 / 0.51	263.8	277.0	195.5	2	50	"	"
4	PB-Cut BC34	34.17	86.65	126,655	49.4	0.19 / 50.4	2616.2	3395.0	2010.0	2281	24.5	"	"
5	PA-UnCut BC35	13.4	33.32	42,795	22.5	0.19 / 77.3	1759.8	2670.0	1227.0	660	30.9	"	"
6	PA-UnCut BC36	17.26	42.53	66,602	19.7	0.19 /80.2	1549.1	2381.0	1056.0	713	30.4	"	"
7	PB-UnCut BC37	22.27	48.24	114,967	10.4	0.16 / 89.4	917.9	1579.0	497.0	690	29.5	"	"
8	PB-UnCut BC38	14.71	35.92	64,789	18.1	0.19 / 81.7	1461.4	2111.0	992.0	736	30.4	"	"

Table 9 shows that libraries prepared with the CRISPR cut generally produced more raw reads and a much larger fraction of them is composed of the desired chimeric reads containing proviral and host DNA. The CRISPR cut libraries also identified a large number of integration sites. The comparison with an Illumina based library prepared from the same timepoint, using ~4ug of template, shows that PCIP can identify more integration sites. This experiment also shows that only libraries with a size distribution that mirrors that observed in the sheared DNA should be sequenced, libraries with a preponderance of shorter fragments mainly represent nonspecific amplification.

Example 4: Identifying genomic insertions and internal variants in HTLV-1

Adult T-cell leukemia (ATL) is an aggressive cancer induced by HTLV-1. It is generally characterized by the presence of a single dominant malignant clone, identifiable by a unique proviral integration site. We and others have developed methods based on ligation mediated PCR and Illumina sequencing to simultaneously identify integration sites and determine the abundance of the corresponding clones [2,7]. We initially applied PCIP-seq to two HTLV-1 induced cases of ATL, both previously analyzed with our Illumina based method (ATL2[7] & ATL100[20]). In ATL100 both methods identify a single dominant clone, with >95% of the reads mapping to a single insertion site on chr18 (Fig. 2a, 2b & Table 8). Using the integration site information, we extracted the PCIP-seq hybrid reads spanning the provirus/host insertion site, uncovering a ~3,600bp deletion within the provirus (Fig. 2c).

In the case of ATL2, PCIP-seq showed three major proviruses located on chr5, chr16 and chr1, each responsible for ~33% of the HTLV-1/host hybrid reads. We had previously established that these three proviruses are in a single clone via examination of the T-cell receptor gene rearrangement[7]. However, it is interesting to note that this was not initially obvious using our Illumina based method as the proviral insertion site on chr1 falls within a repetitive element (LTR) causing many of the reads to map to multiple regions in the genome. If multi mapping reads are filtered out, the chr1 insertion site accounted for 13.7% of the remaining reads, while retaining multi mapping produces values closer to reality (25.4%). In contrast the long reads from PCIP-seq allow unambiguous mapping and closely matched the expected 33% for each insertion site (Fig. 2d), highlighting the advantage long reads have in repetitive regions. Looking at the three proviruses, proviral reads revealed all to be full length. Three de novo mutations were observed in one provirus and a single de novo mutation was identified in the second (Fig. 2e).

Example 5: Insertion sites identified in samples with multiple clones of low abundance

The samples utilized above represent a best-case scenario, with ~100% of cells infected and a small number of major clones. We next applied PCIP-seq to four samples from BLV infected sheep (experimental infection[21]) and three cattle (natural infection) to explore its performance on polyclonal and low proviral load (PVL) samples and compared PCIP-seq to our previously published Illumina method[7]. PCIP-seq revealed all samples to be highly polyclonal (Figure 10 and Table 8) with the number of unique insertion sites identified varying from 172 in the bovine sample 560 (1µg template, PVL 0.644%) to 17,903 in bovine sample 1053 (6µg template, PVL 23.5%). In general, PCIP-seq identified more insertion sites, using less input DNA than our Illumina based method (Table 10).

Table 10. Comparing PCIP-seq to ligation mediated PCR and Illumina sequencing. For the Illumina libraries the template DNA used was 4 µg. For the PCIP-seq it varied between libraries (233=7µg, 221(022016)=4µg, 221(032014)=4µg, 220=2µg, 1439=3µg, 560=1µg, 1053=6µg). >3 signifies insertion sites supported by more than 3 reads after PCR duplicate removal. ILLUMINA = Ligation mediated PCR with Illumina sequencing. U-IS ILL. in PCIP = Unique insertion sites (%) identified in ILLUMINA and also found in PCIP-seq. Correlation Abundance Overlapping IS. Pearson's correlation Abundance = correlation of abundances from proviruses detected in both Illumina and PCIP-seq.

Sample	Insertion sites ILLUMINA	Insertion sites PCIP-seq	U-IS ILL. in PCIP (%)	Pearson Correlation	Insertion sites ILLUMINA (>3)	Insertion sites PCIP-seq (>3)	U-IS ILL. in PCIP (%) (>3)	Raw PCIP-seq reads	Raw Illumina reads
233	1110	5311	81.2	0.949810181	448	2302	85.9	524698	173196
221 (022016)	1122	8023	40.4	0.511939213	74	3546	50	180276	9579
221 (032014)	4473	5374	44.4	0.526457101	1555	1524	34.9	32266	391478
220	915	1352	36.1	0.894732877	401	664	47.6	44876	299554
1439	5784	5773	47.7	0.894732877	1449	3053	63.9	181055	216525
560	379	172	15.8	0.616804459	81	77	33.3	6802	192170
1053	8496	17903	62.0	0.811169919	2196	7777	68.5	367454	219461

Comparison of the results showed a significant overlap between the two methods. When we consider insertion sites supported by more than three reads in both methods (larger clones, more likely to be present in both samples), in the majority of cases >50% of the insertion sites identified in the Illumina data were also observed via PCIP-seq (Table 10). These results show the utility of PCIP-seq for insertion site identification, especially considering the advantages long reads have in repetitive regions of the genome.

Example 6: Identifying SNPs in BLV proviruses

Portions of the proviruses with more than ten supporting reads (PCR duplicates removed) were examined for SNPs with LoFreq[22]. For the four sheep samples, the variants were called relative to the pBLV344 provirus (used to infect the animals). For the bovine samples 1439 and 1053 custom consensus BLV sequences were generated for each and the variants were called in relation to the appropriate reference (SNPs were not called in 560). Across all the samples 3,209 proviruses were examined, 934 SNPs were called and 680 (21%) of the proviruses carried one or more SNPs (Table 11).

Table 11. Numbers of SNPs identified in each sample.

Sample name	Species	PVL	# Insertion sites	# Proviruses examined for SNPs	# Variants detected (AF > 0.6)	# Proviruses with variant (AF > 0.6)	# Positions within proviruses with variant (AF > 0.6)
233	OAR	78.3	5311	789	233	168	136
221 (022016)	OAR	63.0	8023	408	93	79	86
221 (032014)	OAR	16.0	5374	70	6	6	6
220	OAR	3.8	1352	130	50	42	36
1439	BosT	45.0	5773	587	311	211	137
1053	BosT	23.5	17903	1243	241	182	169

We validated 10 BLV SNPs in the ovine samples and 15 in the bovine via clone specific long-range PCR and Illumina sequencing. For Ovine 221, which was sequenced twice over a two-year interval, we identified and validated three instances where the same SNP and provirus were observed at both time points. We noted a small number of positions in the BLV provirus prone to erroneous SNP calls. By comparing allele frequencies from bulk Illumina and Nanopore data these problematic positions could be identified. For example, we observed a number of BLV proviruses in all the samples that had an apparent SNP at position 8213. When we looked at this position in reads mapped to the provirus without first sorting based on insertion site (referred to as bulk) we saw a C called 36 and 38% of the time respectively in the Nanopore data. In the bulk Illumina data, generated from the same sample, we saw the C is called 0% of the time indicating a technical artifact. As a consequence, SNPs from this position were excluded.

Approximately half of the SNPs (47.1% sheep, 51.6% cattle) were found in multiple proviruses. Generally, SNPs found at the same position in multiple proviruses were concentrated in a single individual, indicating their presence in a founder provirus or via a mutation in the very early rounds of viral replication. For example, in animal 233 we found 16 proviruses (provirus inclusion was based on the less stringent criteria of >10 reads covering the position, not filtered for PCR duplicates) carrying a T-to-C transition within the Tax ORF at position 8154, this variant does not change the amino acid. Illumina and Nanopore bulk sequencing from the same sample show C is called at a 2% frequency in Nanopore, while with Illumina C is called at a 1% frequency. This indicates that the SNPs observed in these proviruses are not a technical artifact. Alternatively, a variant may also rise in frequency due to increased fitness of clones carrying a mutation in that position. In this instance, we would expect to see the same position mutated in multiple individuals. One potential example is found in the first base of codon 303 (position 8155) of the viral protein Tax, a potent viral transactivator, stimulator of cellular proliferation and highly immunogenic[23]. A variant was observed at this position in five proviruses for sheep 233 and three for sheep 221 as well as one provirus from bovine 1439 (Fig. 3 a). Using less stringent criteria for the inclusion of a proviral region (>10 reads, not filtered for PCR duplicates) we found 34 proviruses in the ovine and 3 in the bovine carrying a variant in this position. The majority of the variants observed were G-to-A transitions (results in E-to-K amino acid change), however we also observed G-to-T (E-to-STOP) and G-to-C (E-to-Q) transversions. It has been previously shown that the G-to-A mutation abolishes the Tax proteins transactivator activity[23,24]. The repeated selection of variants at this specific position suggests that they reduce viral protein

recognition by the immune system, while preserving the Tax proteins other proliferative properties.

Patterns of provirus-wide APOBEC3G[25] induced hypermutation (G-to-A) were not observed in BLV. However, three proviruses (two from sheep 233 and one in bovine 1053) showed seven or more A-to-G transitions, confined to a ~70bp window in the first half of the U3 portion of the 3'LTR. The pattern of mutation, as well as their location in the provirus suggests the action of RNA adenosine deaminases 1 (ADAR1)[26,27].

Example 7: PCIP-seq identifies BLV structural variants in multiple clones

Proviruses were also examined for structural variants (SVs) using a custom script and via visualization in IGV (see Example 1). Between the sheep and bovine samples, we identified 66 deletions and 3 tandem duplications, with sizes ranging from 15bp to 4,152bp, with a median of 113bp (Table 12).

Table 12: BLV structural variants identified via PCIP-seq

1053

Provirus	Type	Region in BLV	Approx size	Clone specific PCR
1_120275095_120275095	DEL	230-252	22	no
1_147862114_147862122	DEL	2241-2275	34	no
2_106933456_106933462	DEL	7674-7708	34	no
3_6970332_6970339	DEL	5109-6728	1619	no
3_90671155_90671163	DEL	2608-2919	311	no
4_114867583_114867589	DEL	4574-4637	63	no
5_25818093_25818100	DEL	4482-4526	44	no
6_95273607_95273614	DEL	4487-5537	1050	no
6_112133285_112133291	DEL	5217-5368	151	no
10_101509344_101509352	DEL	7324-7425	101	no
12_36183673_36183673	DEL	1808-1835	27	no
13_35328779_35328785	DEL	3679-4603	924	no
15_24605050_24605054	DEL	8136-8162	26	no
16_28380797_28380803	DEL	2984-3895	911	no
17_64277037_64277043	DEL	5418-5636	218	no
20_7882911_7882911	DEL	8111-8137	26	no
20_7882911_7882911	DEL	8230-8340	110	no
21_53434814_53434824	DEL	6854-7130	276	no
21_53434814_53434824	DEL	7202-7246	44	no
22_40343810_40343823	DEL	4629-4838	209	no

1439

Provirus	Type	Region in BLV	Approx size	Clone specific PCR
10_65013091_65013093	DEL	2164-3192	1028	no
1_150385145_150385351	DEL	3451-3474	23	yes
2_121703720_121703726	DEL	5350-5399	49	no
23_39892380_39892560	DEL	2364-2560	196	yes
2_4188067_4188067	DEL	2176-2570	394	no
24_3748146_3748155	DEL	5419-5497	78	no
27_36582809_36582809	DEL	4522-5636	1114	yes
27_36582809_36582809	DEL	1-852	852	yes
4_100234239_100234246	INS	8296-8370	75	yes
5_51456241_51456285	DEL	1-4152	4152	yes
2_124084208_124084213	DEL	391-406	15	yes
3_45576532_45576538	DEL	2316-2336	20	yes
5_95348339_95348346	DEL	8167-8200	33	no
8_112613917_112613964	DEL	4225-6244	2019	no
5_6307451_6307451	INS	3251-3590	338	no

22_48239823_48239830	DEL	2271-2799	528	no
23_41760533_41760533	DEL	8100-8201	101	no
24_22643966_22643974	DEL	6857-7165	308	no
25_33749737_33749744	DEL	4225-4264	39	no
28_28470239_28470248	DEL	4496-5191	695	no
29_25146501_25146508	DEL	3901-5251	1350	no
X_33071616_33071616	DEL	3322-3969	647	no
X_61600607_61600612	DEL	6193-6783	590	no

221 (022016 & 032014)

Provirus	Type	Region in BLV	Approx size	Clone specific PCR
OAR3_128671913_128671921	DEL	4591-4620	30	no
OAR18_26694984_26694991	DEL	5287-5508	222	no
OAR25_25097056_25097063	DEL	2325-4303	1979	yes
OARX_110727773_110727797	DEL	2858-2970	113	no
OARX_78143793_78143801	DEL	3284-6602	3298	yes

221 (032014)

Provirus	Type	Region in BLV	Approx size	Clone specific PCR
OAR14_25755878_25755884	DEL	5846-6486	640	no

221 (022016)

Provirus	Type	Region in BLV	Approx size	Clone specific PCR
OAR1_25125478_25125485	DEL	6237-6255	19	no
OAR1_250672128_250672136	DEL	7365-7389	25	yes
OAR2_73878244_73878251	DEL	237-264	28	no
OAR3_149619110_149619110	DEL	7610-7726	117	no
OAR3_211678275_211678275	DEL	6228-6285	58	no
OAR8_80161637_80161982	DEL	6502-6561	60	yes
OAR13_10090846_10090865	DEL	6484-6561	78	no
OAR16_10037623_10037623	DEL	1287-1396	110	no
OAR21_31148897_31148902	DEL	7292-7544	253	no
OAR24_28280610_28280610	DEL	6807-6828	22	no
OAR2_242159705_242159712	INS	7017-7232	215	yes

233

Provirus	Type	Region in BLV	Approx size	Clone specific PCR
OAR10_34545991_34546003	DEL	5298-5330	32	yes
OAR10_49266255_49266262	DEL	6512-6586	74	yes
OAR14_42146250_42146256	DEL	1658-1724	66	no
OAR16_3998022_3998027	DEL	4479-4706	227	no
OAR19_37466567_37466573	DEL	278-428	150	no
OAR23_14140808_14140814	DEL	3270-5878	2608	no
OAR3_184106381_184106391	DEL	5799-5874	75	no
OAR7_72584331_72584331	DEL	4574-5453	879	no
OAR7_72649090_72649098	DEL	539-629	90	no

We validated 14 of these via clone specific PCR. As seen in Fig. 3b SVs were found throughout the majority of the provirus, encompassing the highly expressed microRNAs[28] as well as the second exon of the constitutively expressed antisense transcript *AS1*[29]. Only two small regions at the 3' end lacked any SVs. More proviruses will need to be examined to see if this pattern holds, but these results again suggest the importance of the 3'LTR and its previously reported interactions with adjacent host genes[7].

Example 8: Identifying HIV-1 integration sites and the associated provirus

Despite the effectiveness of combination antiretroviral therapy (ART) in suppressing HIV-1 replication, cART is not capable of eliminating latently infected cells, ensuring a viral rebound if cART is suspended[30]. This HIV-1 reservoir represents a major obstacle to a HIV cure[31] making its exploration a priority. However, this task is complicated by its elusiveness, with only ~0.1% of $CD4^+$ T cells carrying integrated HIV-1 DNA[32]. To see if PCIP-seq could be applied to these extremely low proviral loads we initially carried out dilution experiments using U1[33], a HIV-1 cell line containing replication competent proviruses[34]. PCIP-seq on undiluted U1 DNA found the major insertion sites on chr2 and chrX (accounting for 47% & 41% of the hybrid reads respectively) and identified the previously reported variants that disrupt Tat function[35] in both proviruses. In the chr2 provirus a T-to-C changes ATG to ACG and the first methionine to a threonine. In the chrX provirus an A-to-T changes CAT to CTT replacing a histidine at position 13 with a leucine. In addition to the two major proviruses we identified an additional ~700 low abundance insertion sites (Table 8) including one on chr19 (0.8%) reported by Symons et al 2017[34] that is actually a product of recombination between the major chrX and chr2 proviruses, and one on chr7 (chr7: 100.5). Identification of the chr7: 100.5 & chr19: 34.9 proviruses as the products of recombination between major chrX and chr2 proviruses was shown by mapping proviral reads from all four proviruses to a full length proviral genome (the sequence (SEQ ID NO:4) of the chrX provirus was used as the reference). This allowed to identify SNPs and sequences derived from respectively, the chr2 and chrX provides. We then serially diluted U1 DNA in Jurkat cell line DNA. PCIP-seq was carried out with 5 µg of template DNA where U1 represents 0.1% and 0.01% of the total DNA. We also processed 5 µg of Jurkat DNA in parallel as a negative control. The three PCIP-seq libraries were prepared using the same guides and primers. Following sequencing and demultiplexing the Jurkat negative control produced 12,137 reads, Jurkat + U1 0.01% produced 234,421 reads and Jurkat + U1 0.1% 252,913 reads. The resultant reads were mapped to the human genome. We were able to detect the major proviruses on chr2 and chrX in both dilutions (Table 8). The reads were also mapped the HIV-1 genome. No reads of pure HIV-1 or chimeric HIV-1/host reads mapping to HIV-1 were observed in the Jurkat negative control (Table 14). In Jurkat + U1 0.01% samples 12.6% of the reads were chimeric HIV-1/host, in Jurkat + U1 0.1% this rose to 43.2%.

Example 9: Identifying full-length and polymorphic endogenous retroviruses in cattle and sheep

ERVs in the genome can be present as full length, complete provirus, or more commonly as solo-LTRs, the products of non-allelic recombination[37]. At the current time

conventional short read sequencing, using targeted or whole genome approaches, cannot distinguish between the two classes. Examining full length ERVs would provide a more complete picture of ERV variation, while also revealing which elements can produce *de novo* ERV insertions. As PCIP-seq targets inside the provirus we can preferentially amplify full length ERVs, opening this type of ERV to study in larger numbers of individuals. As a proof of concept we targeted the class II bovine endogenous retrovirus BERVK2, known to be transcribed in the bovine placenta[38]. We applied the technique to three cattle, of which one (10201e6) was a Holstein suffering from cholesterol deficiency, an autosomal recessive genetic defect recently ascribed to the insertion of a 1.3kb LTR in the *APOB* gene[39]. PCIP-seq clearly identified the *APOB* ERV insertion in 10201e6, whereas no reads were seen mapping to this position in libraries from the other two cattle (Mannequin & 571). In contrast to previous reports[39] PCIP-seq shows it to be a full-length element. We identified a total of 67 ERVs, with 8 present in all three samples (Table 15).

Table 15: Endogenous retroviruses (BERVK2) identified in cattle via PCIP-seq. *LTR matches APOB ERV (BTA11_77.9); #ERV inserted into APOB; Full = Full length ERV; Partial = ERV with large deletion.

#	Approximate location in genome (BTA6)	Provirus name	10201e6	Mannequin	571	Provirus
1	chr1:108,822,892-108,832,262	BTA1_108.8	no	no	YES	Full
2	chr1:140,473,236-140,486,732	BTA1_140.4	YES	no	YES	Full
3	chr2:7,341,443-7,349,776	BTA2_7.3	no	no	YES	Full
4	chr2:68,574,688-68,583,604	BTA2_68.5	YES	no	no	Partial
5	chr2:108,763,340-108,771,071	BTA2_108.7	no	YES	no	Full
6	chr2:136,856,893-136,860,100	BTA2_136.8	YES	no	no	Full
7	chr3:11,025,879-11,032,187	BTA3_11.0	no	YES	no	Full
8	chr3:21,243,379-21,247,173	BTA3_21.24	no	YES	no	Full
9	chr3:21,262,507-21,266,148	BTA3_21.26	no	YES	no	Full
10	chr3:115,305,677-115,313,191	BTA3_115.3	YES	no	no	**Full***
11	chr4:23,529,679-23,538,398	BTA4_23.5	YES	no	no	Partial
12	chr4:106,804,424-106,812,368	BTA4_106.8	no	no	YES	Full
13	chr5:76,505,040-76,518,833	BTA5_76.5	YES	YES	YES	Full
14	chr6:19,795,982-19,804,772	BTA6_19.7	YES	YES	YES	Full
15	chr6:33,664,998-33,674,349	BTA6_33.6	YES	no	no	Full
16	chr6:93,979,584-93,984,028	BTA6_93.9	YES	YES	YES	Partial
17	chr7:18,507,208-18,514,234	BTA7_18.5	no	YES	no	Partial
18	chr7:62,318,935-62,329,558	BTA7_62.3	YES	no	no	Full
19	chr7:109,501,965-109,512,061	BTA7_109.5	YES	no	YES	Full
20	chr8:16,410,224-16,424,259	BTA8_16.4	YES	no	YES	Full
21	chr8:37,357,029-37,369,016	BTA8_37.3	no	YES	no	Full
22	chr8:67,963,331-67,972,754	BTA8_67.9	no	YES	no	Full
23	chr8:81,237,785-81,244,766	BTA8_81.2	YES	YES	no	Full
24	chr9:15,412,806-15,418,477	BTA9_15.4	YES	no	no	Partial
25	chr9:83,082,008-83,092,749	BTA9_83.0	YES	no	no	Full

#	Approximate location in genome (BTA6)	Provirus name	10201e6	Mannequin	571	Provirus
26	chr9:84,257,434-84,262,548	BTA9_84.2	YES	no	no	Full
27	chr9:101,949,614-101,957,434	BTA9_101.9	YES	YES	no	Full
28	chr10:71,920,524-71,928,975	BTA10_71.9	YES	no	no	Full
29	chr10:87,425,735-87,443,841	BTA10_87.4	YES	YES	YES	Partial
30	chr11:50,592,847-50,606,524	BTA11_50.5	YES	no	YES	Full
31	chr11:61,788,705-61,792,024	BTA11_61.7	no	YES	no	Full
32	chr11:77,955,413-77,963,724	BTA11_77.9	YES	no	no	**Full#**
33	chr12:72,978,039-72,985,406	BTA12_72.9	YES	YES	no	Full
34	chr12:74,723,248-74,731,915	BTA12_74.7	YES	YES	no	Partial
35	chr15:9,435,764-9,439,369	BTA15_9.4	YES	YES	YES	Full
36	chr16:10,720,162-10,727,571	BTA16_10.7	YES	no	no	Full
37	chr16:13,308,596-13,315,659	BTA16_13.3	YES	no	no	Partial
38	chr16:28,504,653-28,536,456	BTA16_28.5	YES	no	YES	Full
39	chr18:27,619,893-27,626,348	BTA18_27.6	YES	no	YES	Partial
40	chr18:27,715,161-27,722,285	BTA18_27.7	no	no	YES	Full
41	chr18:50,368,602-50,378,304	BTA18_50.3	YES	YES	YES	Full
42	chr18:60,211,168-60,220,590	BTA18_60.2	YES	YES	YES	Partial
43	chr18:61,691,367-61,697,347	BTA18_61.6	YES	no	YES	Full
44	chr19:5,180,841-5,189,334	BTA19_5.1	YES	no	no	Partial
45	chr19:22,014,748-22,025,138	BTA19_22.0	YES	no	no	Full
46	chr19:51,039,969-51,101,363	BTA19_51.0	no	YES	YES	Partial
47	chr20:15,283,426-15,290,599	BTA20_15.2	YES	no	no	Full
48	chr20:55,126,259-55,134,120	BTA20_55.1	no	YES	no	Full
49	chr21:1,241,740-1,256,399	BTA21_1.2	YES	YES	YES	Partial
50	chr21:2,303,211-2,307,834	BTA21_2.3	no	YES	no	Full
51	chr21:4,133,180-4,142,631	BTA21_4.1	no	no	no	Full
52	chr21:18,634,068-18,645,042	BTA21_18.6	no	YES	no	Full
53	chr22:160,456-166,792	BTA22_160.4	no	no	YES	Full
54	chr23:41,312,657-41,328,100	BTA23_41.3	YES	no	no	Full
55	chr23:52,329,640-52,337,577	BTA23_52.3	YES	no	no	Full
56	chr24:12,819,683-12,824,449	BTA24_12.6	YES	YES	no	Partial
57	chr24:53,067,680-53,078,844	BTA24_53.0	no	no	YES	Full
58	chr25:20,428,960-20,444,963	BTA25_20.4	no	no	no	Full
59	chr26:50,606,858-50,616,960	BTA26_50.6	YES	no	no	Full
60	chr27:14,146,146-14,156,627	BTA27_14.1	no	YES	no	Full
61	chr28:17,575,320-17,582,731	BTA28_17.5	YES	no	no	Full
62	chr29:39,631,808-39,639,476	BTA29_39.6	YES	no	no	Full
63	chrX:27,723,875-27,732,458	BTAX_27.7	no	YES	no	Full
64	chrX:30,183,463-30,187,122	BTAX_30.1	YES	no	no	Partial
65	chrX:36,260,818-36,264,888	BTAX_36.2	YES	no	no	Partial
66	chrX:43,949,278-43,960,449	BTAX_43.9	no	no	YES	Full
67	chrX:47,314,044-47,327,526	BTAX_47.3	no	no	YES	Full

We validated three ERVs via long range PCR and Illumina sequencing. We did not find any with an identical sequence to the *APOB* ERV, although the ERV BTA3_115.3 has an identical LTR sequence, highlighting that the sequence of the LTR cannot be used to infer the complete sequence of the ERV.

We also adapted PCIP-seq to amplify the Ovine endogenous retrovirus Jaagsiekte sheep retrovirus (enJSRV), a model for retrovirus-host co-evolution[40]. The PCIP-seq

reads were mapped to the reference genome (OAR3) where sequences matching enJSRV had been masked out, this preventing reads from multiple proviruses mapping to these positions. Hybrid reads in the unique flanking sequence allowed us to determine the sequence of the proviruses present at these locations. Using two sheep (220 & 221) as template we identified a total of 48 enJSRV proviruses, (33 in 220 and 38 in 221, with 22 common to both) and of these ~54% were full length (Table 16).

Table 16: Endogenous retroviruses (enJSRV) identified in sheep via PCIP-seq. Full = Full length ERV; Partial = ERV with large deletion.

	Approximate location in genome (OAR3)	ERV name	**220**	**221**	provirus
1	chr1:57,132,178-57,139,903	OAR1_57.13	no	YES	Full
2	chr1:86,065,652-86,091,348	OAR1_86.0	YES	YES	Full
3	chr1:129,489,883-129,502,056	OAR1_129.4	no	YES	Full
4	chr1:220,250,002-220,258,800	OAR1_220.2	YES	YES	Full
5	chr1:240,077,458-240,092,905	OAR1_240.0	YES	YES	Partial
6	chr1:253,739,233-253,756,582	OAR1_253.7	YES	YES	Partial
7	chr2:196,585,537-196,593,010	OAR2_196.5	YES	no	Full
8	chr3:39,261,134-39,285,428	OAR3_39.2	YES	YES	Full
9	chr3:39653898-39656987	OAR3_39.6	YES	YES	Partial
10	chr3:151,767,643-151,783,037	OAR3_151.7	YES	YES	Partial
11	chr3:182,538,937-182,555,692	OAR3_182.5	YES	no	Full
12	chr4:40,485,410-40,504,790	OAR4_40.4	YES	YES	Full
13	chr4:77,416,611-77,428,510	OAR4_77.4	YES	YES	Partial
14	chr5:7,744,521-7,756,178	OAR5_7.74	YES	YES	Partial
15	chr5:64,916,815-64,926,920	OAR5_64.9	YES	no	Partial
16	chr5:73,009,027-73,018,771	OAR5_73.0	YES	no	Full
17	chr6:5,400,881-5,410,594	OAR6_5.4	no	YES	Full
18	chr6:6,789,991-6,858,767	OAR6_6.7	YES	YES	Partial
19	chr6:26,968,086-26,977,558	OAR6_26.9	no	YES	Full
20	chr8:2,974,531-2,988,179	OAR8_2.9	YES	YES	Partial
21	chr8:49,483,598-49,499,241	OAR8_49.4	YES	YES	Partial
22	chr9:48,096,442-48,105,912	OAR9_48.0	no	YES	Full
23	chr9:89,743,769-89,752,495	OAR9_89.7	no	YES	Partial
24	chr10:70,892,072-70,919,960	OAR10_70.8	YES	no	Partial
25	chr11:32,085,050-32,095,786	OAR11_32.0	YES	YES	Full
26	chr13:5,676,353-5,686,765	OAR13_5.6	no	YES	Full
27	chr13:16,714,529-16,726,069	OAR13_16.7	YES	YES	Full
28	chr13:37,514,438-37,529,955	OAR13_37.5	YES	YES	Full
29	chr13:66022872-66031772	OAR13_66.0	YES	no	Full
30	chr14:13,811,039-13,844,103	OAR14_13.8	YES	YES	Partial
31	chr14:15,011,370-15,043,076	OAR14_15.0	YES	YES	Partial
32	chr14:56,232,971-56,236,157	OAR14_56.2	YES	YES	Full
33	chr14:57,491,683-57,503,056	OAR14_57.4	no	YES	Partial
34	chr14:57,605,121-57,623,737	OAR14_57.6	YES	YES	Partial
35	chr15:10,864,017-10,870,430	OAR15_10.8	no	YES	Full
36	chr17:48,876,178-48,887,208	OAR17_48.8	no	YES	Full
37	chr18:1,738,143-1,751,356	OAR18_1.7	no	YES	Partial
38	chr18:67,778,281-67,799,930	OAR18_67.7	YES	YES	Full

	Approximate location in genome (OAR3)	ERV name	**220**	**221**	provirus
39	chr19:52,665,989-52,689,785	OAR19_52.6	YES	YES	Partial
40	chr20:433,819-443,901	OAR20_0.4	YES	no	Full
41	chr20:1,237,366-1,250,699	OAR20_1.2	no	YES	Partial
42	chr20:27,598,593-27,615,677	OAR20_27.5	no	YES	Full
43	chr21:6,694,384-6,709,701	OAR21_6.6	YES	no	Partial
44	chr22:46,781,990-46,790,196	OAR22_46.7	no	YES	Full
45	chr26:8,253,764-8,265,010	OAR26_8.2	no	YES	Full
46	chrX:3,690,949-3,701,009	OARX_3.6	YES	no	Full
47	chrX:62,939,566-62,949,333	OARX_62.9	YES	YES	Partial
48	chrX:78,127,416-78,132,398	OARX_78.1	YES	no	Partial

We validated seven proviruses via long-range PCR and Illumina sequencing.

Example 10: Extending PCIP-seq to human papillomaviruses (HPV)

The majority of HPV infections clear or are suppressed within 1–2 years[41], however a minority evolve into cancer, and these are generally associated with integration of the virus into the host genome. This integration into the host genome is not part of the viral lifecycle and the breakpoint in the viral genome can occur at any point across is 8kb circular genome[16]. As a consequence the part of the viral genome found at the virus host breakpoint varies considerably, making the identifying of integration sites difficult using existing approaches[16]. The long reads employed by PCIP-seq mean that even when the breakpoint is a number of kb away from the position targeted by primers we should still capture the integration site. As a proof of concept, we applied PCIP-seq to two HPV18 positive cases, (HPV18_PX and HPV18_PY) using 4 µg of DNA extracted from Pap smear material. We identified 55 integration sites in HPV18_PX and 19 integration sites in HPV18_PY (Table 17).

Table 17: HPV integration sites identified in patients HPV18_PX and HPV18_PY. Estimated read count refers to number of reads after PCR duplicates have been removed, see https://github.com/GIGA-AnimalGenomics-BLV/PCIP/blob/master/README.md

Patient	ID	Estimated read count	Overlapping Gene	geneID	Notes
HPV18_PX	chr1:201993711-201993711	1	RNPEP	ENSG00000176393	
HPV18_PX	chr1:54070808-54070808	1	TCEANC2	ENSG00000116205	
HPV18_PX	chr1:74339164-74339164	2	FPGT-TNNI3K	ENSG00000259030	
HPV18_PX	chr11:72988358-72988358	6	FCHSD2	ENSG00000137478	
HPV18_PX	chr12:124528897-124528897	5	NCOR2	ENSG00000196498	
HPV18_PX	chr12:62430096-62430096	3	NA	NA	
HPV18_PX	chr12:88750111-88750111	2	NA	NA	

HPV18_PX	chr13:32401471-32401471	1	N4BP2L1	ENSG00000139597	
HPV18_PX	chr13:59883976-59883976	1	DIAPH3	ENSG00000139734	
HPV18_PX	chr13:70017637-70017637	1	KLHL1	ENSG00000150361	
HPV18_PX	chr13:96145444-96145444	1	HS6ST3	ENSG00000185352	
HPV18_PX	chr16:35696743-35696743	4	NA	NA	
HPV18_PX	chr16:46391666-46391666	15	NA	NA	
HPV18_PX	chr16:60839237-60839237	3	NA	NA	
HPV18_PX	chr17:50736162-50736162	1	LUC7L3	ENSG00000108848	
HPV18_PX	chr17:71945217-71945217	1	NA	NA	
HPV18_PX	chr18:33256597-33256597	2	CCDC178	ENSG00000166960	
HPV18_PX	chr2:175176252-175176252	1	NA	NA	
HPV18_PX	chr2:184979785-184979785	1	NA	NA	
HPV18_PX	chr2:222973976-222973976	1	NA	NA	
HPV18_PX	chr20:26724089-27697774	1	NA	NA	Virus in satellite repeat
HPV18_PX	chr20:59882951-59882951	4	SYCP2	ENSG00000196074	
HPV18_PX	chr21:31443081-31443081	5	TIAM1	ENSG00000156299	
HPV18_PX	chr21:8210410-8210516	6	FP671120.3	ENSG00000280800	
HPV18_PX	chr21:8225927-8228889	9	FP671120.1	ENSG00000278996	
HPV18_PX	chr21:8393406-8393551	9	FP236383.2	ENSG00000280614	
HPV18_PX	chr21:8437761-8437761	9	FP236383.3	ENSG00000281181	
HPV18_PX	chr21:8453856-8454775	19	NA	NA	
HPV18_PX	chr3:141177260-141177260	1	NA	NA	
HPV18_PX	chr3:183646815-183646815	5	KLHL24	ENSG00000114796	
HPV18_PX	chr3:52477576-52477615	67	NISCH	ENSG00000010322	
HPV18_PX	chr3:52491989-52492028	67	NISCH	ENSG00000010322	
HPV18_PX	chr3:52564151-52564190	75	SMIM4	ENSG00000168273	
HPV18_PX	chr4:113196089-113196089	3	ANK2	ENSG00000145362	
HPV18_PX	chr4:118149173-118149173	2	NDST3	ENSG00000164100	
HPV18_PX	chr4:125160196-125160196	2	NA	NA	
HPV18_PX	chr4:8361851-8361851	1	NA	NA	
HPV18_PX	chr5:85159333-85159333	2	NA	NA	
HPV18_PX	chr6:12217019-12217019	1	NA	NA	
HPV18_PX	chr6:58604926-59721758	1	NA	NA	Virus in satellite repeat
HPV18_PX	chr6:60995120-60995120	4	NA	NA	
HPV18_PX	chr6:72218404-72218404	3	RIMS1	ENSG00000079841	
HPV18_PX	chr6:7655460-7655460	6	NA	NA	
HPV18_PX	chr7:55353950-55353950	10	NA	NA	
HPV18_PX	chr7:63798384-63798384	3	NA	NA	
HPV18_PX	chr7:7812181-7812181	4	AC007161.3	ENSG00000283549	
HPV18_PX	chr7:98111088-98111088	1	LMTK2	ENSG00000164715	

HPV18_PX	chr8:119801685-119801685	13	TAF2	ENSG00000064313	
HPV18_PX	chr8:2564068-2564068	1	NA	NA	
HPV18_PX	chr8:93515097-93515097	1	LINC00535	ENSG00000246662	
HPV18_PX	chr8:9886409-9886409	2	NA	NA	
HPV18_PX	chr9:12503146-12503146	1	NA	NA	
HPV18_PX	chr9:128458663-128458663	1	ODF2	ENSG00000136811	
HPV18_PX	chrX:19414286-19414286	1	MAP3K15	ENSG00000180815	
HPV18_PX	chrX:41675298-41675299	1	CASK	ENSG00000147044	

HPV18_PY	chr5:37774016-37774016	2	NA	NA	
HPV18_PY	chr7:64329003-64329003	2	ZNF736	ENSG00000234444	
HPV18_PY	chr4:184039889-184039889	2	NA	NA	
HPV18_PY	chr18:108534-108534	2	NA	NA	
HPV18_PY	chr3:59699600-59699600	1	NA	NA	
HPV18_PY	chr4:90546531-90546531	1	CCSER1	ENSG00000184305	
HPV18_PY	chr5:146985347-146985347	1	PPP2R2B	ENSG00000156475	
HPV18_PY	chr6:41200232-41200232	1	TREML2	ENSG00000112195	
HPV18_PY	chr6:113561576-113561576	1	NA	NA	
HPV18_PY	chr1:107169512-107169512	1	NTNG1	ENSG00000162631	
HPV18_PY	chr1:218361256-218361256	1	TGFB2	ENSG00000092969	
HPV18_PY	chr3:52563123-52563123	1	SMIM4	ENSG00000168273	
HPV18_PY	chr9:15686595-15686595	1	CCDC171	ENSG00000164989	
HPV18_PY	chr9:137787856-137787856	1	AL590627.1	ENSG00000255585	
HPV18_PY	chr10:6703026-6703026	1	AL158210.2	ENSG00000285743	
HPV18_PY	chr10:23788794-23788794	1	KIAA1217	ENSG00000120549	
HPV18_PY	chr10:91570894-91570894	1	NA	NA	
HPV18_PY	chr11:97096506-97096506	1	NA	NA	
HPV18_PY	chr19:35339090-35339090	1	CD22	ENSG00000012124	

In HPV18_PY the vast majority of the reads only contained HPV sequences, the integration sites identified were defined by single reads, suggesting little or no clonal expansion (Table 8). In HPV18_PX most integration sites were again defined by a single read, however there were some exceptions (Table 17). HPV18_PX had integrated copies of HPV18 on chr21 and chr3 (Figure 5). Both integration sites contained multiple copies of the HPV genome. The most striking of these was a cluster of what appeared to be three integration sites located within the region chr3:52477576-52564190 (Fig. 5a). The unusual pattern of read coverage combined with the close proximity of the virus-host breakpoints indicated that these three integration sites were connected. Long range PCR

with primers spanning positions α-β and α-γ, showed that a genomic rearrangement had occurred in this clonally expanded cell (Fig. 5a). Regions α and β are adjacent to one another with HPV integrated between, however PCR also showed regions α and γ to be adjacent to one another, again with the HPV genome integrated between (Fig. 5b). The sequence of the virus found between α-β looks to be derived from the α-γ virus as it shares a breakpoint and is slightly shorter (Fig. 5b). This complex arrangement suggests that this rearrangement was generated via the recently described 'looping' integration mechanism[16,42]. The α and β breakpoints fall within exons of the *NISCH* gene while the γ breakpoint falls within exon 27 of *PBRM1* (Fig. 5c), a gene previously shown to be a cancer driver in renal carcinoma[43] and intrahepatic cholangiocarcinomas[44]. This patient was classified by histology as having atypical glandular cells and a follow up three months later was classified as a high grade CIN3.The PCIP-seq method was applied to DNA from leftover Pap smears, assaying 29 HPV18 and 42 HPV16 positive cases. The majority of the samples had been classified by cytology as Atypical squamous cells of undetermined significance (ASC-US). In both, episomal HPV was the most common finding. We found that the reads generated from episomal HPV can be used to generate a consensus sequence for HPV and as shown in Figure 6 it is possible to examine the phylogenetic relationships between the isolates.

As regards HPV integrations, we identified six patients where integration is associated with a pronounced clonal expansion, four, including HPV18_PX, were infected with HPV18 and two with HPV16.

The second patient had an integration of HPV18 within an intron of LRRC49 (histology = low grade squamous intraepithelial lesion). From the next two clonally expanded integrations (both HPV18), samples from two time points were available. The first had an integration in the LAPTM4B gene, the integration was found in both samplings and in the second it appears that episomal HPV18 has been cleared (Figure 7a, b). (Histology, 1st sample = atypical squamous cells cannot exclude HSIL, 2nd sampling upgraded to High Grade Squamous Intraepithelial Lesion, HSIL).

The last clonally expanded integrations were found in a seventy-one-year-old patient, integration was observed in three different positions in the genome, all were observed in two samplings 5 months apart (Figure 7c, d) (Both time points, histology = atypical squamous cell of undetermined significance). All the integrated copies of HPV18 had intact E6 and E7 genes (both are cancer driver genes and are deregulated when HPV integrates).

As regards HPV16, we identified two samples with clonally expanded integrations. The first was observed in a 53-year-old with a low-grade squamous intraepithelial lesion, the HPV16 genome had integrated ~2.5kb upstream of the KRT5 gene. No episomal HPV16 DNA was observed in this sample. The integrated HPV genome contains a ~3kb deletion that does not overlap with the E6 and E7 genes. The second HPV16 sample has an integration in intron 4 of the POFUT1 gene. Again, the inserted viral genome contains a large deletion (~5.5kb) that does not overlap with E6 and E7. In contrast to the other HPV16 sample the majority (~75%) of the HPV16 reads in this patient were still derived from episomal HPV16.

Discussion

In the present report we describe how PCIP-seq can be utilized to identify insertion sites while also sequencing parts of, and in some cases the entire associated provirus, and confirm this methodology is effective with a number of different retroviruses as well as HPV. For insertion site identification, the method was capable of identifying more than ten thousand BLV insertion sites in a single sample, using ~4µg of template DNA. Even in samples with a PVL of 0.66%, it was possible to identify hundreds of insertion sites with only 1µg of DNA as template. The improved performance of PCIP-seq in repetitive regions further highlights its utility, strictly from the standpoint of insertion site identification. In addition to its application in research, high throughput sequencing of retrovirus insertion sites has shown promise as a clinical tool to monitor ATL progression[20]. Illumina based techniques require access to a number of capital-intensive instruments. In contrast PCIP-seq libraries can be generated, sequenced and analyzed with the basics found in most molecular biology labs, moreover, preliminary results are available just minutes after sequencing begins[45]. As a consequence, the method may have use in a clinical context to track clonal evolutions in HTLV-1 infected individuals, especially as the majority of HTLV-1 infected individuals live in regions of the world with poor biomedical infrastructure[46].

One of the common issues raised regarding Oxford Nanopore data is read accuracy. Early versions of the MinION had read identities of less than 60%[47], however the development of new pores and base calling algorithms make read identities of ~90% achievable[48]. Accuracy can be further improved by generating a consensus from multiple reads, making accuracies of ~99.4%[48] possible. Recently Greig et al[49] compared the performance of Illumina and Oxford Nanopore technologies for SNP identification in two isolates of *Escherichia coli.* They found that after accounting for variants observed at 5-

methylcytosine motif sequences only ~7 discrepancies remained between the platforms. It should be noted that as PCIP-seq sequences PCR amplified DNA, errors generated by base modifications will be avoided. Despite these improvements in accuracy, Nanopore specific errors can be an issue at some positions. Comparison with Illumina data is helpful in the identification of problematic regions and custom base calling models may be a way to improve accuracy in such regions[48]. Additionally, PCIP-seq libraries could equally be sequenced using long reads on the Pacific Biosciences platform or via 10X Genomics linked reads on Illumina if high single molecule accuracy is required[17]. In the current study we focused on SNPs observed in clonally expanded BLV proviruses. For viruses such as HIV-1, which have much lower proviral loads, more caution will be requited as the majority of proviral sequences will be generated from single provirus, making errors introduced by PCR more of an issue.

When analyzing SNPs from BLV the most striking result was the presence of the recurrent mutations at the first base of codon 303 in the viral protein Tax, a central player in the biology of both HTLV-1[46] and BLV[50]. It has previously been reported that this mutation causes an E-to-K amino acid substitution which ablates the transactivator activity of the Tax protein[23]. Collectively, these observations suggest this mutation confers an advantage to clones carrying it, possibly contributing to immune evasion, while retaining Tax protein functions that contribute to clonal expansion. However, there is a cost to the virus as this mutation prevents infection of new cells due to the loss of Tax mediated transactivation of the proviral 5'LTR making it an evolutionary dead end. It will be interesting to see if PCIP-seq can provide a tool to identify other examples of variants that increase the fitness of the provirus in the context of an infected individual but hinder viral spread to new hosts. Additionally, the technique could be used to explore the demographic features of the proviral population within and between hosts, how these populations evolve over time and how they vary.

A second notable observation is the cluster of A-to-G transitions observed within a ~70bp window in the 3'LTR. Similar patterns have been ascribed to ADAR1 hypermutation in a number of viruses[26], including the close BLV relatives HTLV-2 and simian T-cell leukemia virus type 3 (STLV-3)[51]. Given the small number of hypermutated proviruses observed, it appears to be a minor source of variation in BLV, although it will be interesting to see it this holds for different retroviruses and at different time points during infection.

In the current study we focused our analysis on retroviruses and ERVs. However, as this methodology is potentially applicable to a number of different targets we extended its use to HPV as a proof of concept. It is estimated that HPV is responsible for >95% of cervical carcinoma and ~70% of oropharyngeal carcinoma[52]. While infection with a high-risk HPV strain (HPV16 & HPV18) is generally necessary for the development of cervical cancer, it is not sufficient and the majority of infections resolve without adverse consequences[41]. The use of next-generation sequencing has highlighted the central role HPV integration plays in driving the development of cervical cancer[16]. Our results show that PCIP-seq can be applied to identify HPV integration sites in early precancerous samples. This opens up the possibility of generating a more detailed map of HPV integrations as well as potentially providing a biomarker to identify HPV integrations on the road to cervical cancer.

Other potential applications include determining the insertion sites and integrity of retroviral vectors[54] and detecting transgenes in genetically modified organisms. We envision that in addition to the potential applications outlined above many other novel targets/questions could be addressed using this method.

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CLAIMS

1. A method for detecting an integration pattern of human papillomavirus (HPV) in genomic DNA of a subject, said method comprising:

(a) fragmenting genomic DNA isolated from a sample of the subject;

(b) circularizing the DNA fragments to generate circular DNA;

(c) removing non-circularized DNA fragments;

(d) linearizing the circular DNA using an RNA-guided DNA endonuclease and at least one guide RNA or at least one pool of guide RNAs, which target a region in the viral genome, to generate linearized DNA molecules;

(e) amplifying the linearized DNA molecules by an inverse amplification reaction using a pair of primers arranged about and oriented outwardly with respect to the linearization site;

(f) sequencing the amplified DNA;

(g) mapping the sequenced DNA to human genomic DNA sequence; and

(h) optionally mapping the sequenced DNA to the HPV genome.

2. The method according to claim 1, wherein the genomic DNA is fragmented in DNA fragments having an average size of about the HPV genome size.

3. The method according to claim 1 or 2, wherein the amplification reaction comprises long range PCR.

4. The method according to any one of claims 1 to 3, wherein:

a first portion of the circular DNA is linearized using a first guide RNA or a first pool of guide RNAs that target a first region of the viral DNA to generate a first set of linearized DNA molecules; and

a second portion of the circular DNA is linearized using a second guide RNA or a second pool of guide RNAs that target a second region of the viral DNA to generate a second set of linearized DNA molecules,

wherein the first region and the second region of the viral DNA do not overlap.

5. The method according to any one of claims 1 to 4, wherein the linearized DNA molecules are amplified using tailed primers, followed by a second amplification using a set of indexing primers to allow multiplexed sequencing of the amplified DNA.

6. The method according to any one of claims 1 to 5, wherein the sample comprises cervical or vaginal epithelial cells, such as wherein the sample is a pap smear, or wherein the sample comprises oropharyngeal epithelial cells, such as wherein the sample is an oropharyngeal swab.

7. The method according to any one of claims 1 to 6, wherein the HPV is a high-risk HPV strain, preferably a HPV strain 18 or a HPV strain 16.

8. The method according to any one of claims 1 to 7, wherein the at least one guide RNA or the at least one pool of guide RNAs target a region of the viral DNA comprising E6 gene and/or E7 gene.

9. The method according to any one of claims 1 to 8, wherein the HPV is a HPV strain 18 and wherein:

- the first guide RNA or the first pool of guide RNAs to generate the first set of linearized DNA molecules comprises at least one, preferably at least two, and more preferably all three guide RNAs selected from the group consisting of: a guide RNA comprising the targeting domain of SEQ ID NO:232, a guide RNA comprising the targeting domain of SEQ ID NO:233, and a guide RNA comprising the targeting domain of SEQ ID NO:234;
- the second guide RNA or the second pool of guide RNAs to generate the second set of linearized DNA molecules comprises at least one, preferably at least two, and more preferably all three guide RNAs selected from the group consisting of: a guide RNA comprising the targeting domain of SEQ ID NO:235, a guide RNA comprising the targeting domain of SEQ ID NO:236 and a guide RNA comprising the targeting domain of SEQ ID NO:237,

wherein the T in the targeting domains is replaced by U in the guide RNAs;

- the first set of linearized DNA molecules are amplified using a primer pair comprising a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO: 120 (ctccaacgacgcagagaaacac) and a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO:121 (ggattcaacggtttctggcacc); and/or
- the second set of linearized DNA molecules are amplified using a primer pair comprising a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO: 122 (ttttggttcaggctggattgcg) and a primer comprising, consisting

essentially of or consisting of the sequence set forth in SEQ ID NO:123 (agaatacacacagctgccaggt).

10. The method according to any one of claims 1 to 8, wherein the HPV is a HPV strain 16 and wherein:

- the first guide RNA or the first pool of guide RNAs to generate the first set of linearized DNA molecules comprises at least one, preferably at least two, and more preferably all three guide RNAs selected from the group consisting of: a guide RNA comprising the targeting domain of SEQ ID NO:238, a guide RNA comprising the targeting domain of SEQ ID NO:239, and a guide RNA comprising the targeting domain of SEQ ID NO:240;
- the second guide RNA or the second pool of guide RNAs to generate the second set of linearized DNA molecules comprises at least one, preferably at least two, and more preferably all three guide RNAs selected from the group consisting of: a guide RNA comprising the targeting domain of SEQ ID NO:241, a guide RNA comprising the targeting domain of SEQ ID NO:242 and a guide RNA comprising the targeting domain of SEQ ID NO:243,

wherein the T in the targeting domains is replaced by U in the guide RNAs;

- the first set of linearized DNA molecules are amplified using a primer pair comprising a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO:124 (AACCGGACAGAGCCCATTACAA) and a primer comprising, consisting essentially of or consisting of SEQ ID NO:125 (AGTCATATACCTCACGTCGCAGT); and/or
- the second set of linearized DNA molecules are amplified using a primer pair comprising a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO: 126 (ACTGGCTTTGGTGCTATGGACT) and a primer comprising, consisting essentially of or consisting of SEQ ID NO:127 (CAAACCAGCCGCTGTGTATCTG).

11. A kit for detecting an integration pattern of human papillomavirus (HPV) in genomic DNA of a subject according to the method of any one of claims 1 to 10, said kit comprising:

- at least one first guide RNA or at least one first pool of guide RNAs, which target a first region in the viral genome, preferably wherein said first region of the viral DNA comprises E6 gene and/or E7 gene; and/or, preferably and,

-a pair of primers arranged about and oriented outwardly with respect to a first linearization site in the viral genome defined by said at least one first guide RNA or at least first one pool of guide RNAs.

12. The kit for detecting an integration pattern of human papillomavirus (HPV) in genomic DNA of a subject according to claim 11, said kit further comprising:
- at least one second guide RNA or at least one second pool of guide RNAs, which target a second region of the viral DNA, wherein said second region of the viral DNA does not overlap with the first region; and/or, preferably and,
- a pair of primers arranged about and oriented outwardly with respect to a second linearization site in the viral genome defined by said at least one second guide RNA or at least one second pool of guide RNAs.

13. The kit according to claim 11 or 12 further comprising a DNA polymerase for long range PCR.

14. The kit according to any one of claims 11 to 13 further comprising an RNA-guided DNA endonuclease.

15. The kit according to any one of claims 11 to 14 for detecting an integration pattern of a HPV strain 18 wherein:
- the first guide RNA or the first pool of guide RNAs comprise at least one, preferably at least two, and more preferably all three guide RNAs selected from the group consisting of: a guide RNA comprising the targeting domain of SEQ ID NO:232, a guide RNA comprising the targeting domain of SEQ ID NO:233, and a guide RNA comprising the targeting domain of SEQ ID NO:234;
- the second guide RNA or the second pool of guide RNAs comprises at least one, preferably at least two, and more preferably all three guide RNAs selected from the group consisting of: a guide RNA comprising the targeting domain of SEQ ID NO:235, a guide RNA comprising the targeting domain of SEQ ID NO:236 and a guide RNA comprising the targeting domain of SEQ ID NO:237,
wherein the T in the targeting domains is replaced by U in the guide RNAs;
- a primer pair comprising a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO:120 and a primer comprising, consisting essentially of or consisting of SEQ ID NO:121; and/or
- a primer pair comprising a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO: 122 and a primer comprising, consisting essentially of or consisting of SEQ ID NO:123.

16. The kit according to any one of claims 11 to 14 for detecting an integration pattern of a HPV strain 16 comprising:

- the first guide RNA or the first pool of guide RNAs comprises at least one, preferably at least two, and more preferably all three guide RNAs selected from the group consisting of: a guide RNA comprising the targeting domain of SEQ ID NO:238, a guide RNA comprising the targeting domain of SEQ ID NO:239, and a guide RNA comprising the targeting domain of SEQ ID NO:240;
- the second guide RNA or the second pool of guide RNAs comprises at least one, preferably at least two, and more preferably all three guide RNAs selected from the group consisting of: a guide RNA comprising the targeting domain of SEQ ID NO:241, a guide RNA comprising the targeting domain of SEQ ID NO:242 and a guide RNA comprising the targeting domain of SEQ ID NO:243,

wherein the T in the targeting domains is replaced by U in the guide RNAs;

- a primer pair comprising a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO:124 and a primer comprising, consisting essentially of or consisting of SEQ ID NO:125; and/or
- a primer pair comprising a primer comprising, consisting essentially of or consisting of the sequence set forth in SEQ ID NO: 126 and a primer comprising, consisting essentially of or consisting of SEQ ID NO:127.

17. A method for monitoring the progression of a human papillomavirus (HPV) infection in a subject comprising:

- detecting an integration pattern of human papillomavirus (HPV) in genomic DNA isolated from a sample of the subject according to the method of any one of claims 1 to 10; and
- comparing said integration pattern with an integration pattern of HPV in genomic DNA isolated from a sample of the subject at an earlier point in time.

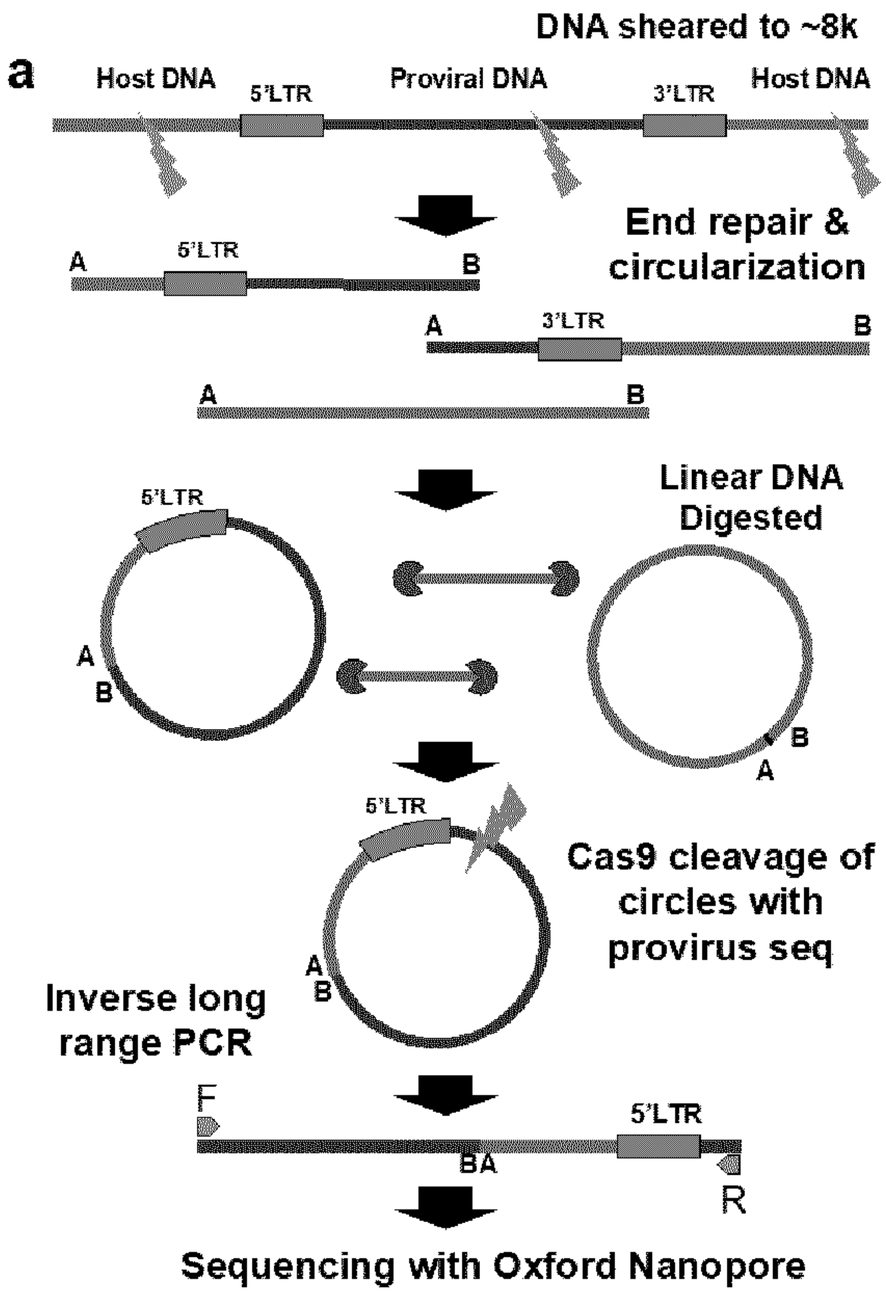
18. A method for assessing a risk of having or developing a cancer in a subject comprising:

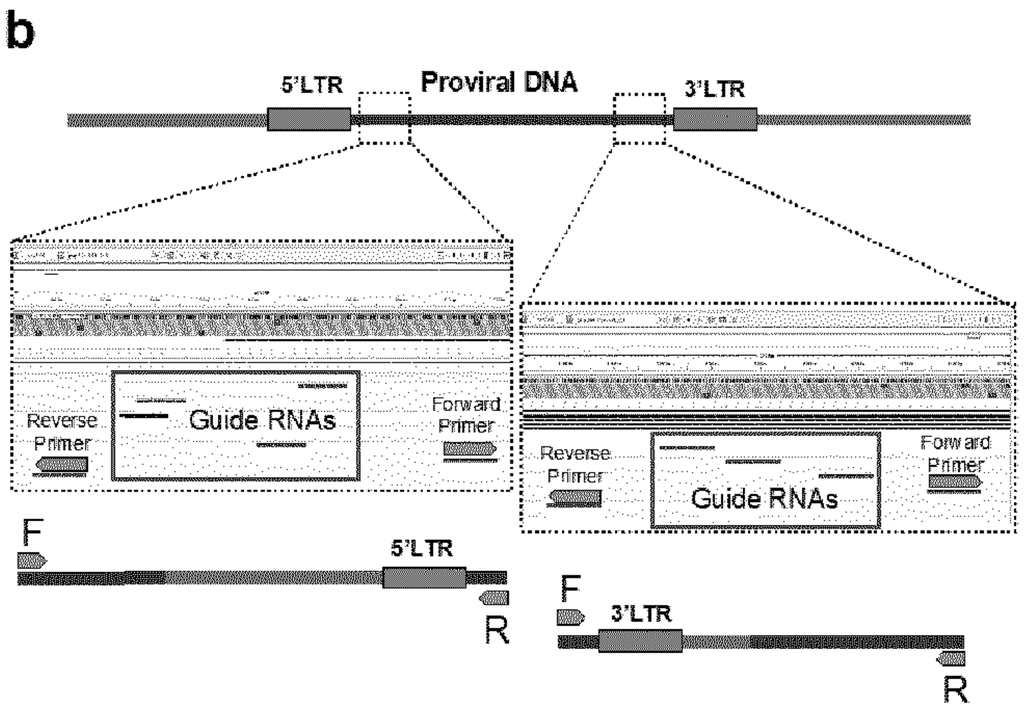
- detecting an integration pattern of human papillomavirus (HPV) in genomic DNA of the subject according to the method of any one of claims 1 to 10; and
- determining whether the integration pattern predisposes the subject to cancer or cancer development.

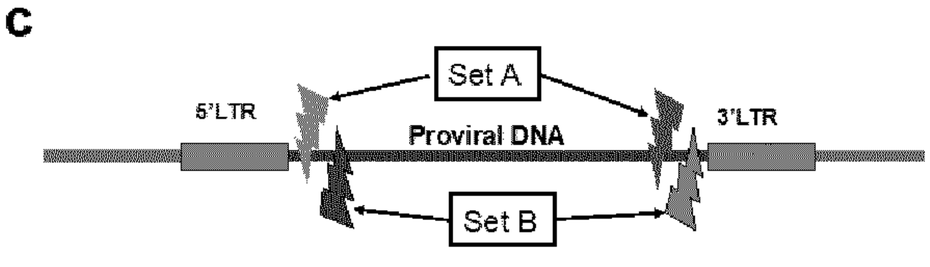
19. The method according to claim 18, wherein said cancer is cervical carcinoma or an oropharyngeal carcinoma.

20. The method according to claim 18 or 19, further comprising a step of determining whether the integration pattern is indicative of clonal expansion.

FIG. 1


a
DNA sheared to ~8k
Host DNA
5'LTR
Proviral DNA
3'LTR
Host DNA
End repair & circularization
A
B
Linear DNA Digested
Cas9 cleavage of circles with provirus seq
Inverse long range PCR
F
R
BA
Sequencing with Oxford Nanopore


b
5'LTR
Proviral DNA
3'LTR
Reverse Primer
Guide RNAs
Forward Primer
F
R


C
Set A
Set B
5'LTR
Proviral DNA
3'LTR

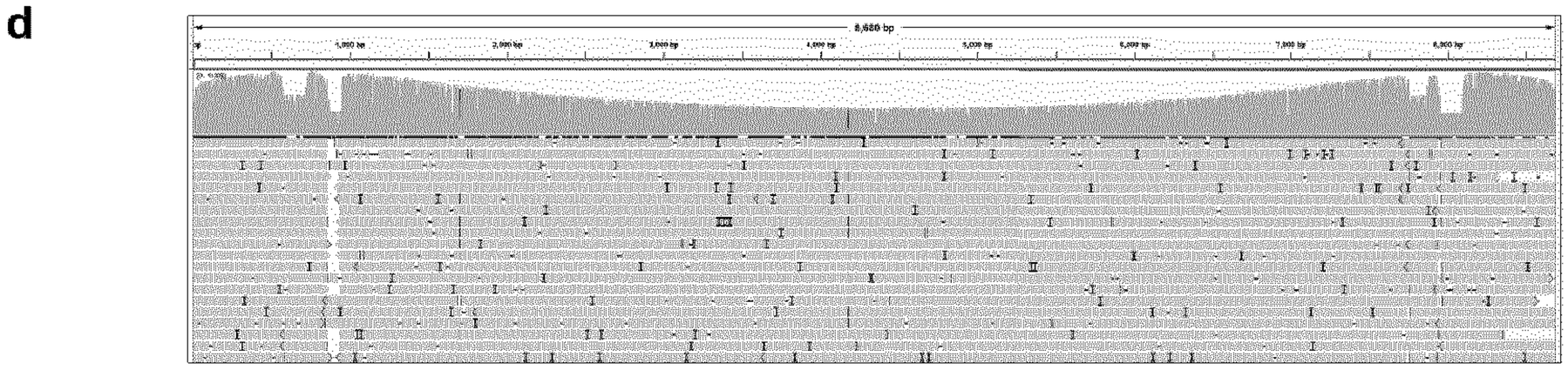
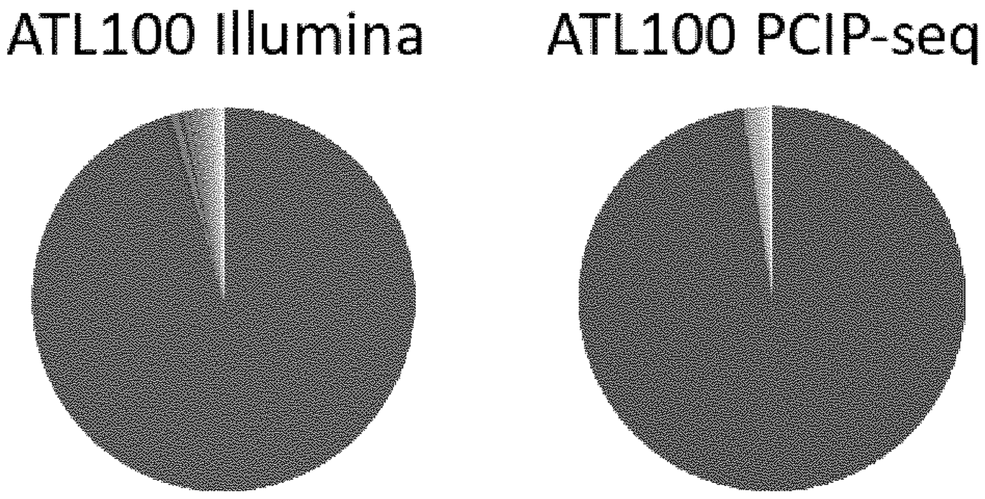
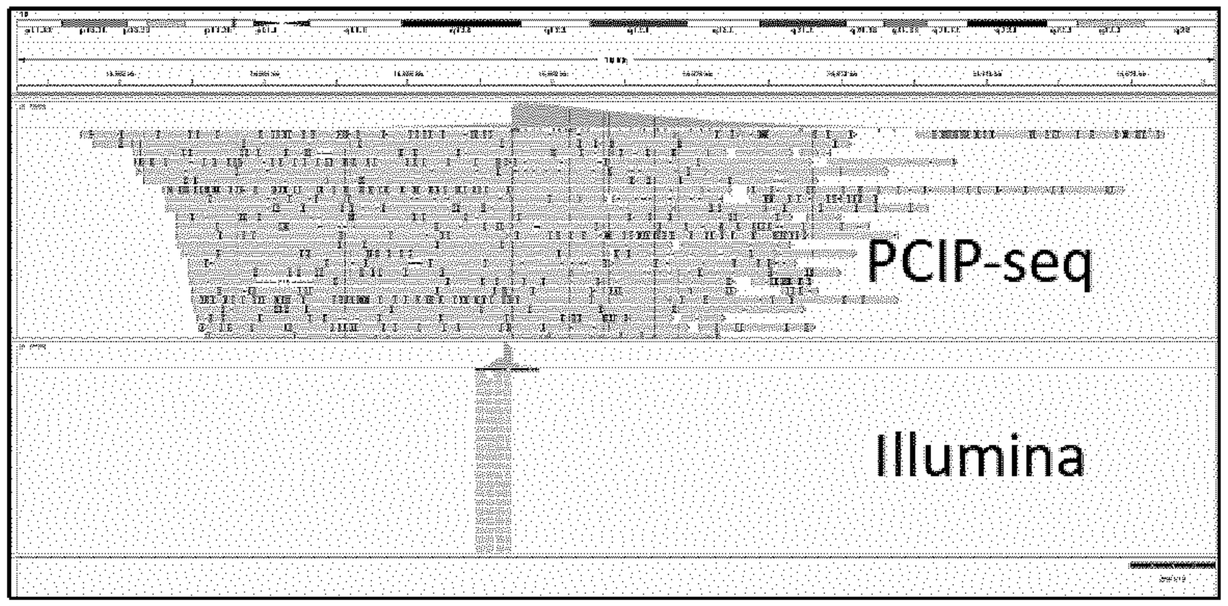

d
8,680 bp

FIG. 2

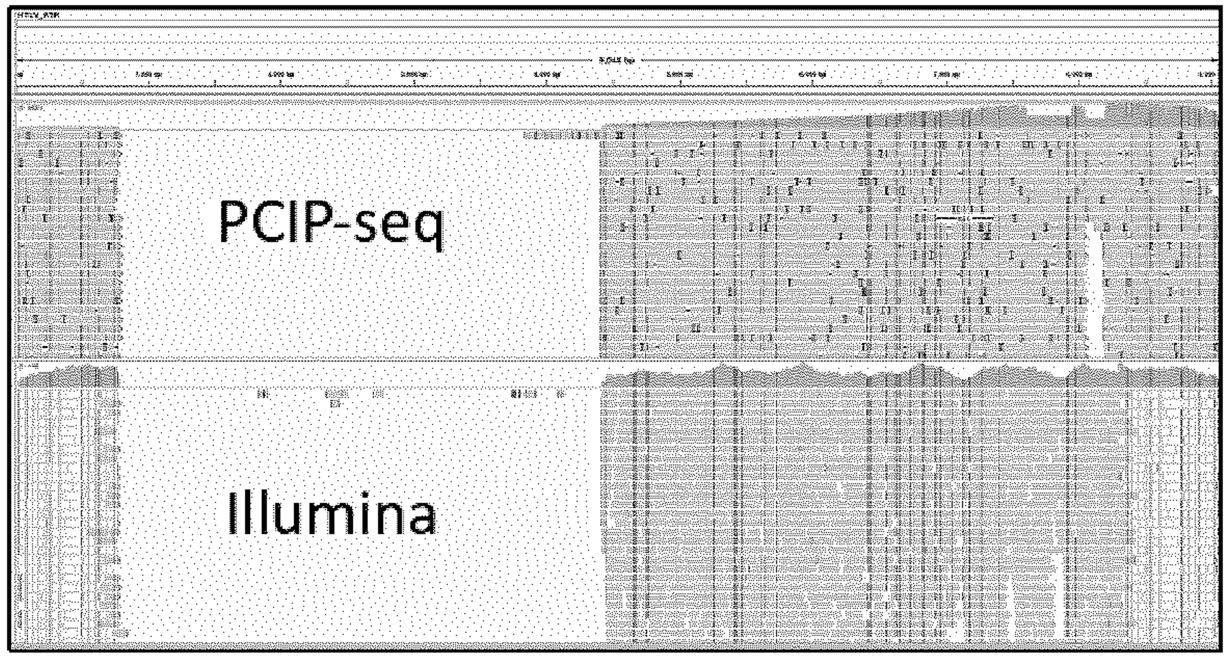
a


ATL100 Illumina
ATL100 PCIP-seq

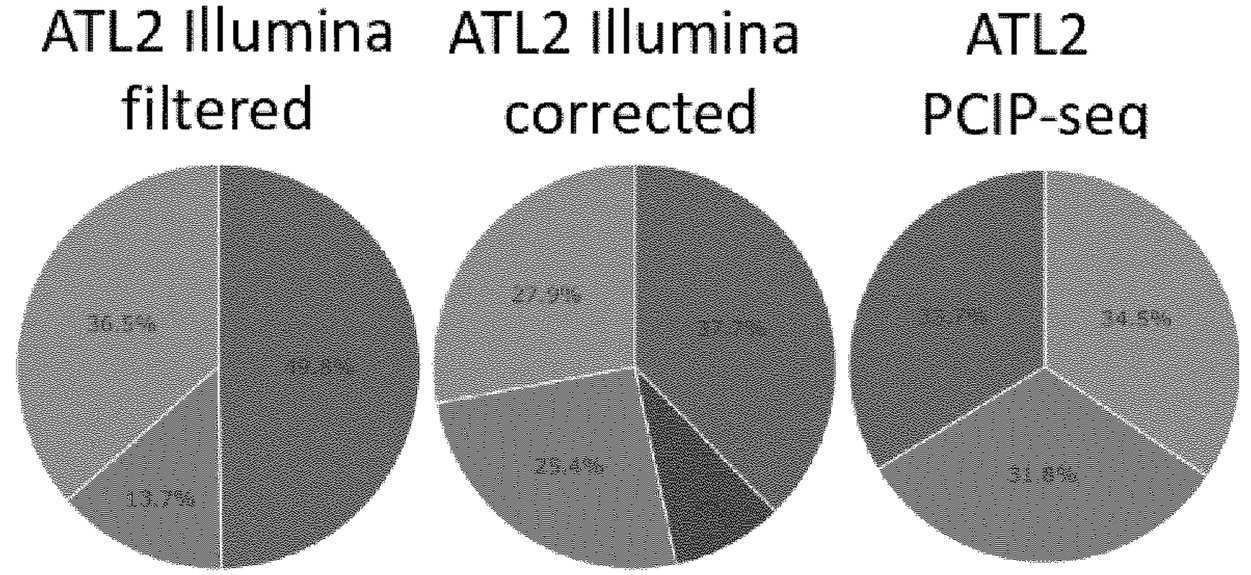
b


PCIP-seq
Illumina

c


PCIP-seq
Illumina

d


ATL2 Illumina filtered
ATL2 Illumina corrected
ATL2 PCIP-seq
36.5%
49.8%
13.7%
27.9%
37.7%
25.4%
33.7%
34.5%
31.8%

e

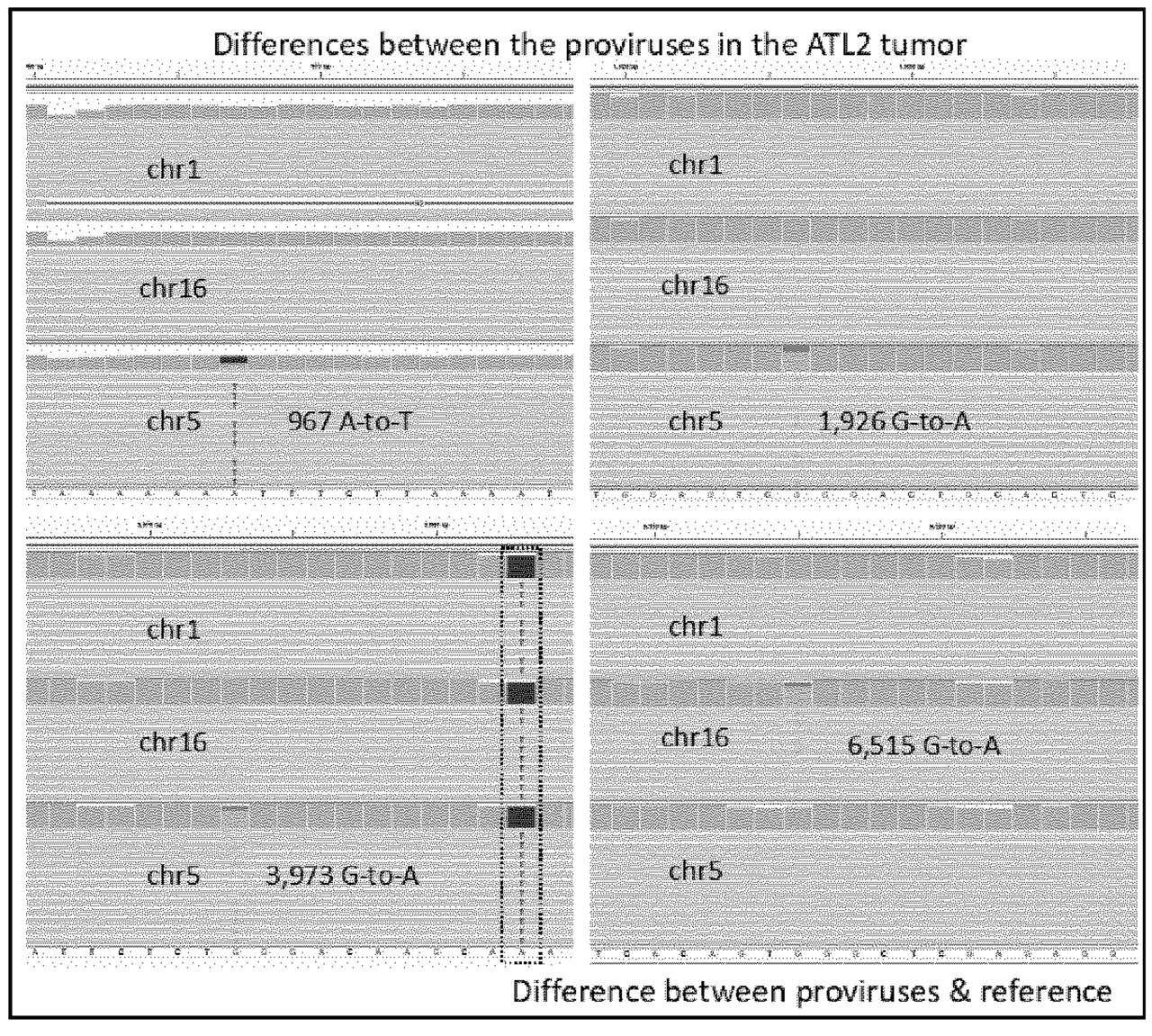
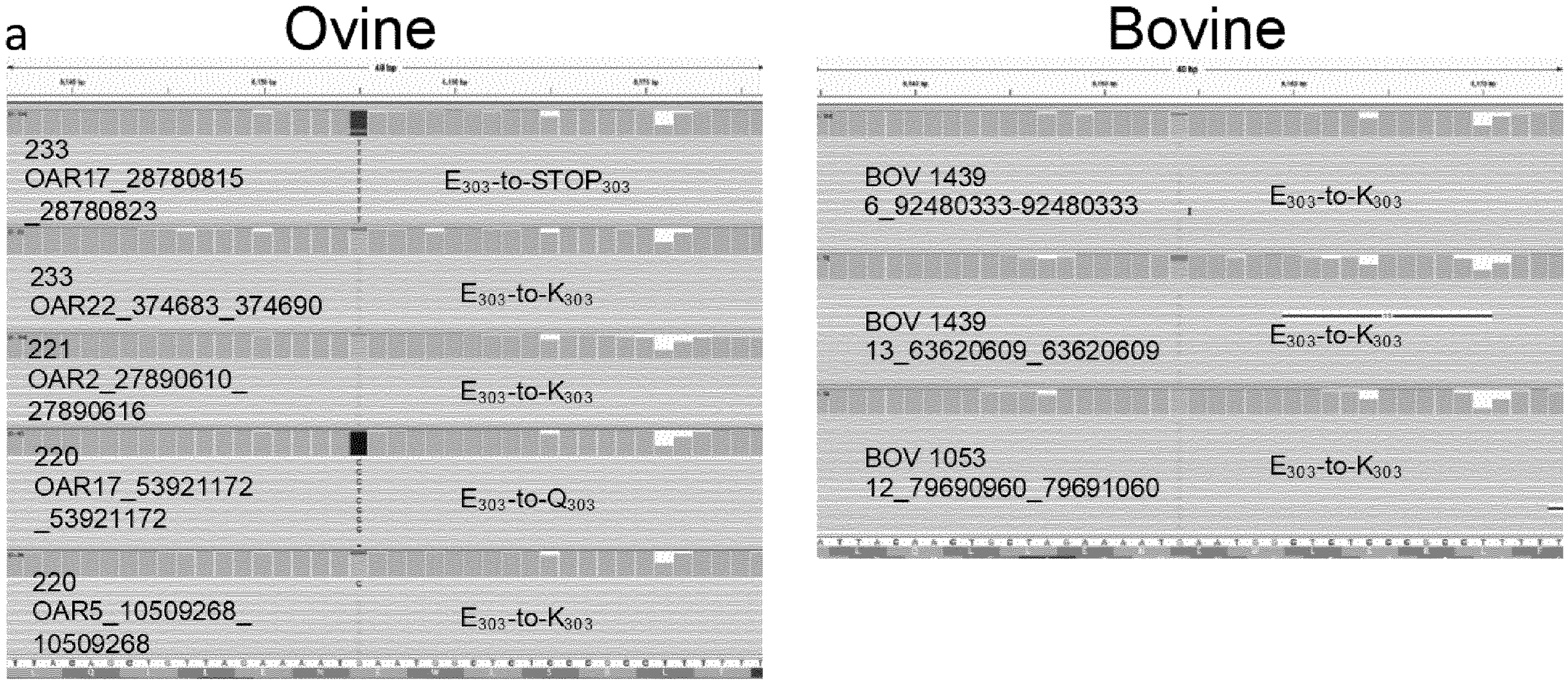

Differences between the proviruses in the ATL2 tumor
chr1
chr16
chr5
967 A-to-T
chr1
chr16
chr5
1,926 G-to-A
chr1
chr16
chr5
3,973 G-to-A
chr1
chr16
chr5
6,515 G-to-A
Difference between proviruses & reference

FIG. 3

a


Ovine
233
OAR17_28780815
_28780823
E303-to-STOP303
233
OAR22_374683_374690
E303-to-K303
221
OAR2_27890610_
27890616
E303-to-K303
220
OAR17_53921172
_53921172
E303-to-Q303
220
OAR5_10509268_
10509268
E303-to-K303
Bovine
BOV 1439
6_92480333-92480333
E303-to-K303
BOV 1439
13_63620609_63620609
E303-to-K303
BOV 1053
12_79690960_79691060
E303-to-K303

b

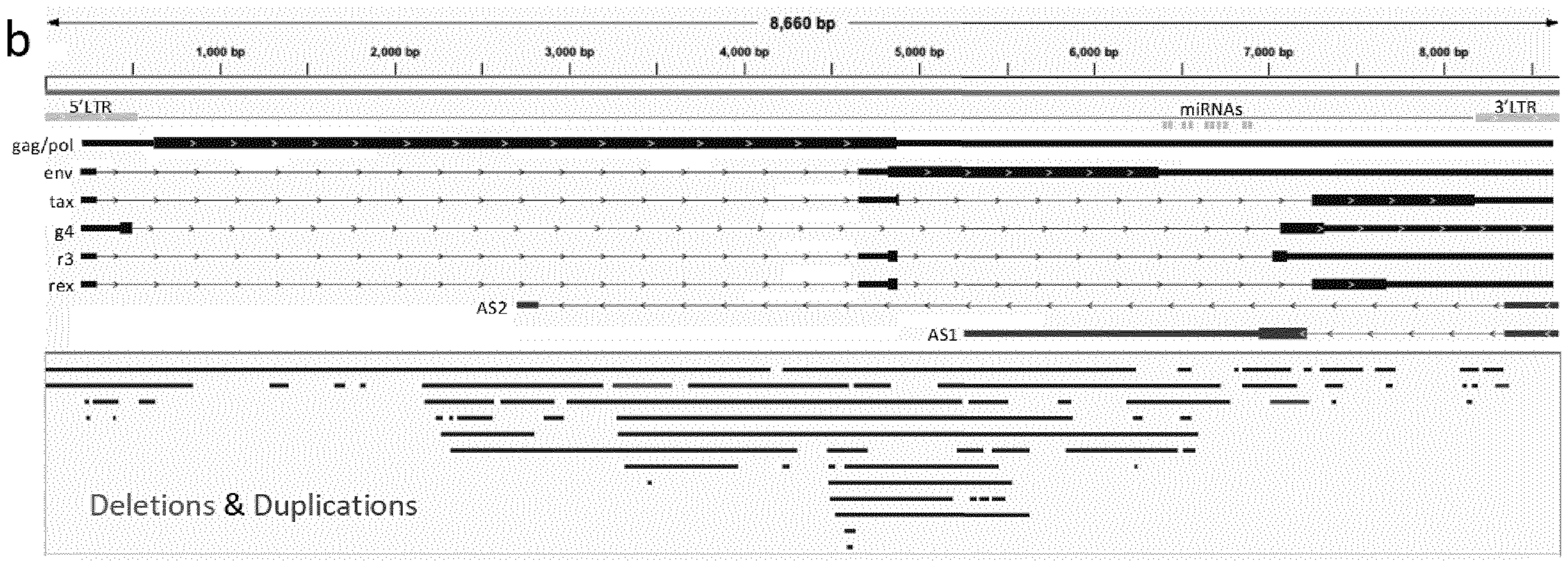

8,660 bp
1,000 bp
2,000 bp
3,000 bp
4,000 bp
5,000 bp
6,000 bp
7,000 bp
8,000 bp
5'LTR
miRNAs
3'LTR
gag/pol
env
tax
g4
r3
rex
AS2
AS1
Deletions & Duplications

FIG. 5

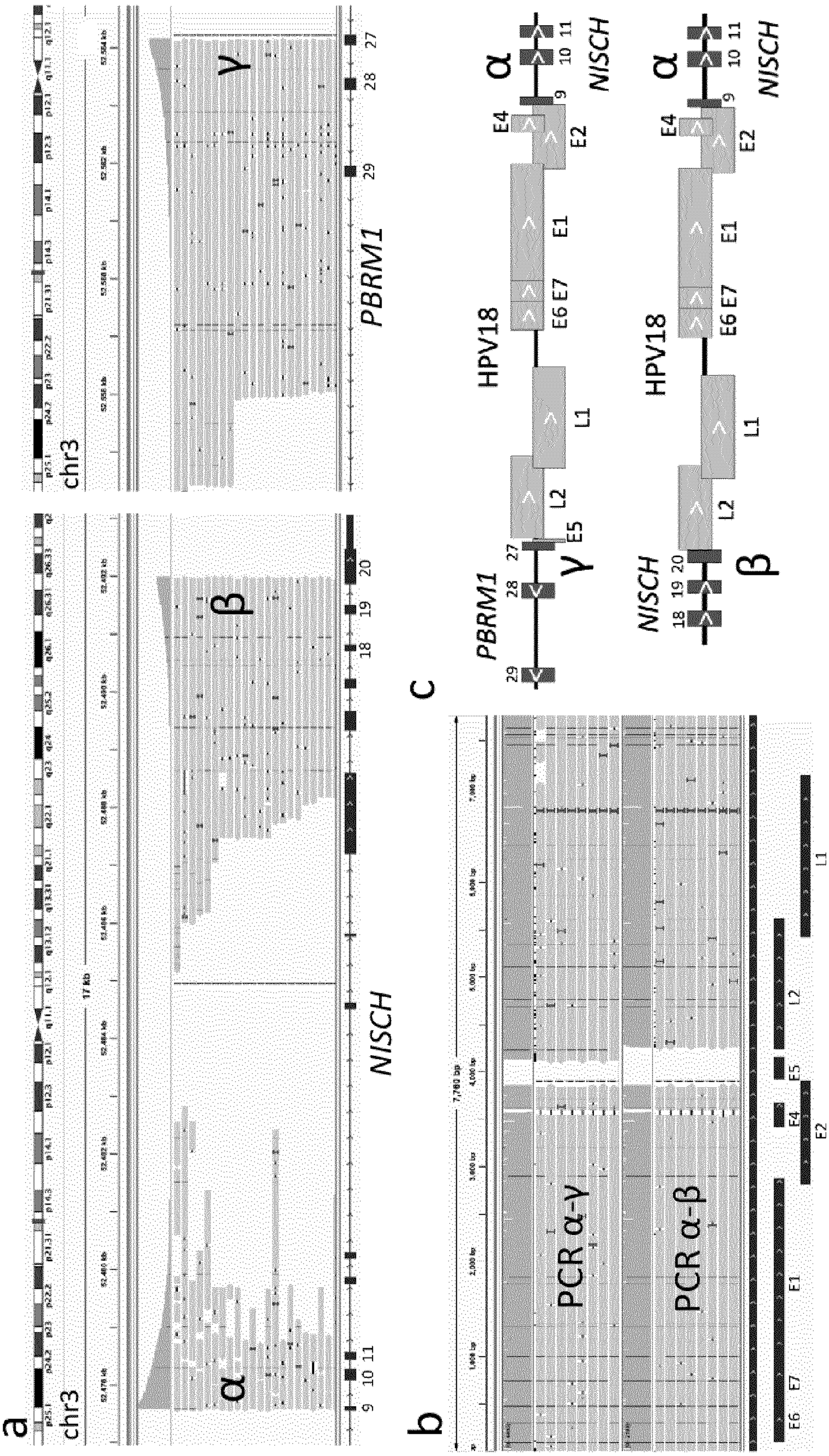

a
chr3
α
9 10 11
NISCH
b
PCR α-γ
PCR α-β
E6 E7 E1
c
HPV18
E2 E4 E5 L2 L1
β
NISCH
18 19 20
γ
PBRM1
29 28 27
chr3
PBRM1
HPV18
E4 E2 E1 E6 E7 L1 L2 E5
α
9 10 11
NISCH

FIG. 6

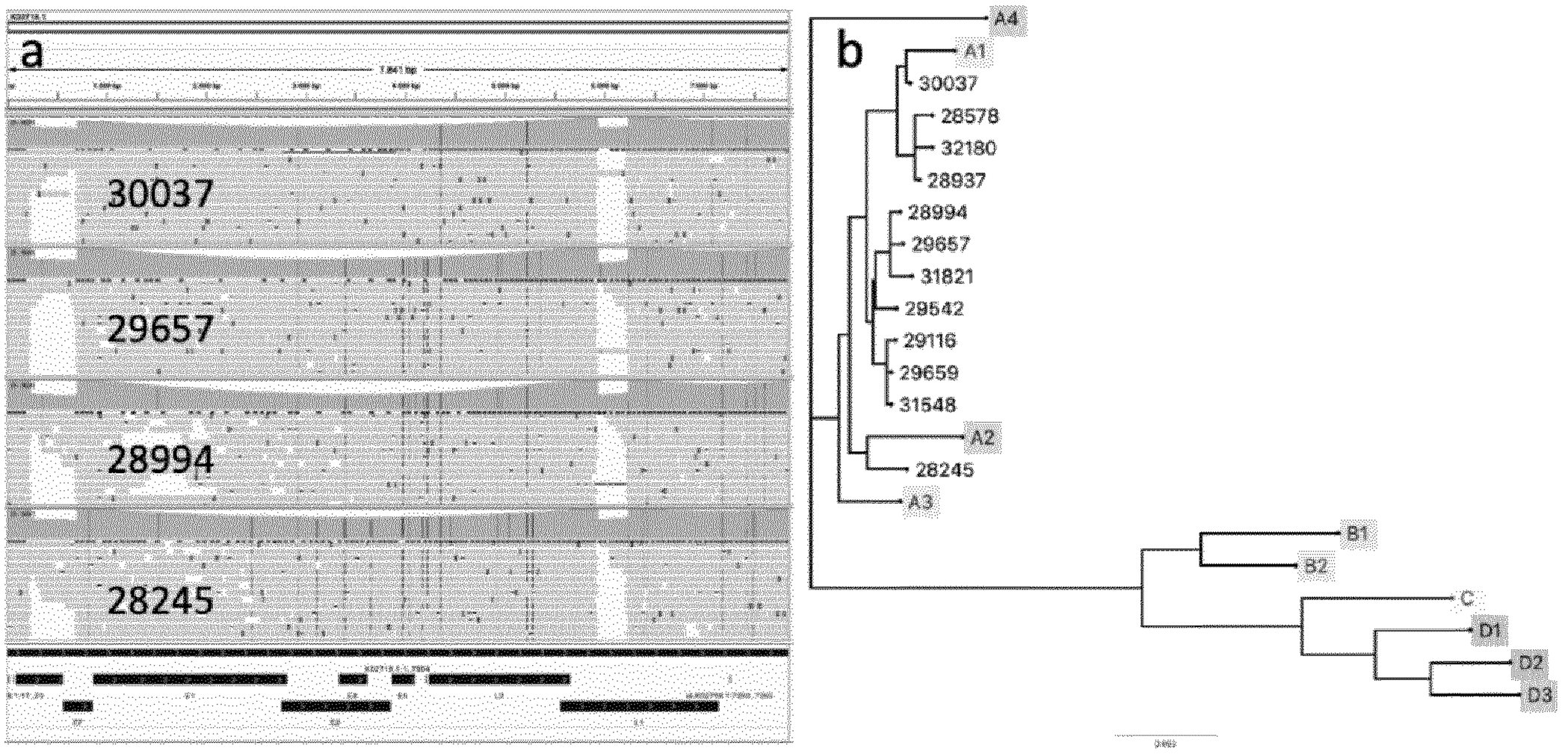

a
30037
29657
28994
28245
b
A4
A1
30037
28578
32180
28937
28994
29657
31821
29542
29116
29659
31548
A2
28245
A3
B1
B2
C
D1
D2
D3

FIG. 7

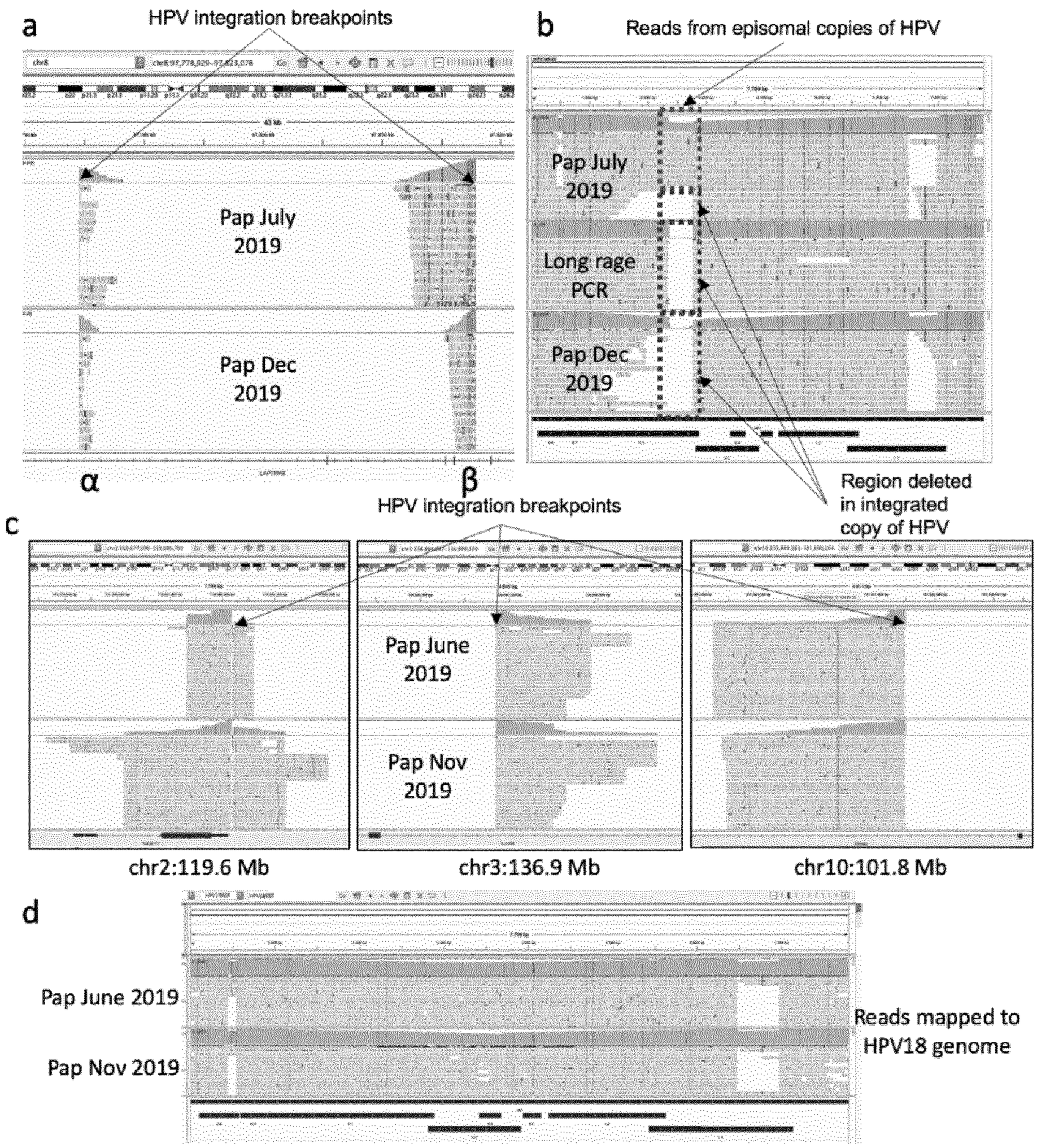

a
HPV integration breakpoints
Pap July 2019
Pap Dec 2019
α
β
b
Reads from episomal copies of HPV
Pap July 2019
Long rage PCR
Pap Dec 2019
Region deleted in integrated copy of HPV
HPV integration breakpoints
c
Pap June 2019
Pap Nov 2019
chr2:119.6 Mb
chr3:136.9 Mb
chr10:101.8 Mb
d
Pap June 2019
Pap Nov 2019
Reads mapped to HPV18 genome

FIG. 8

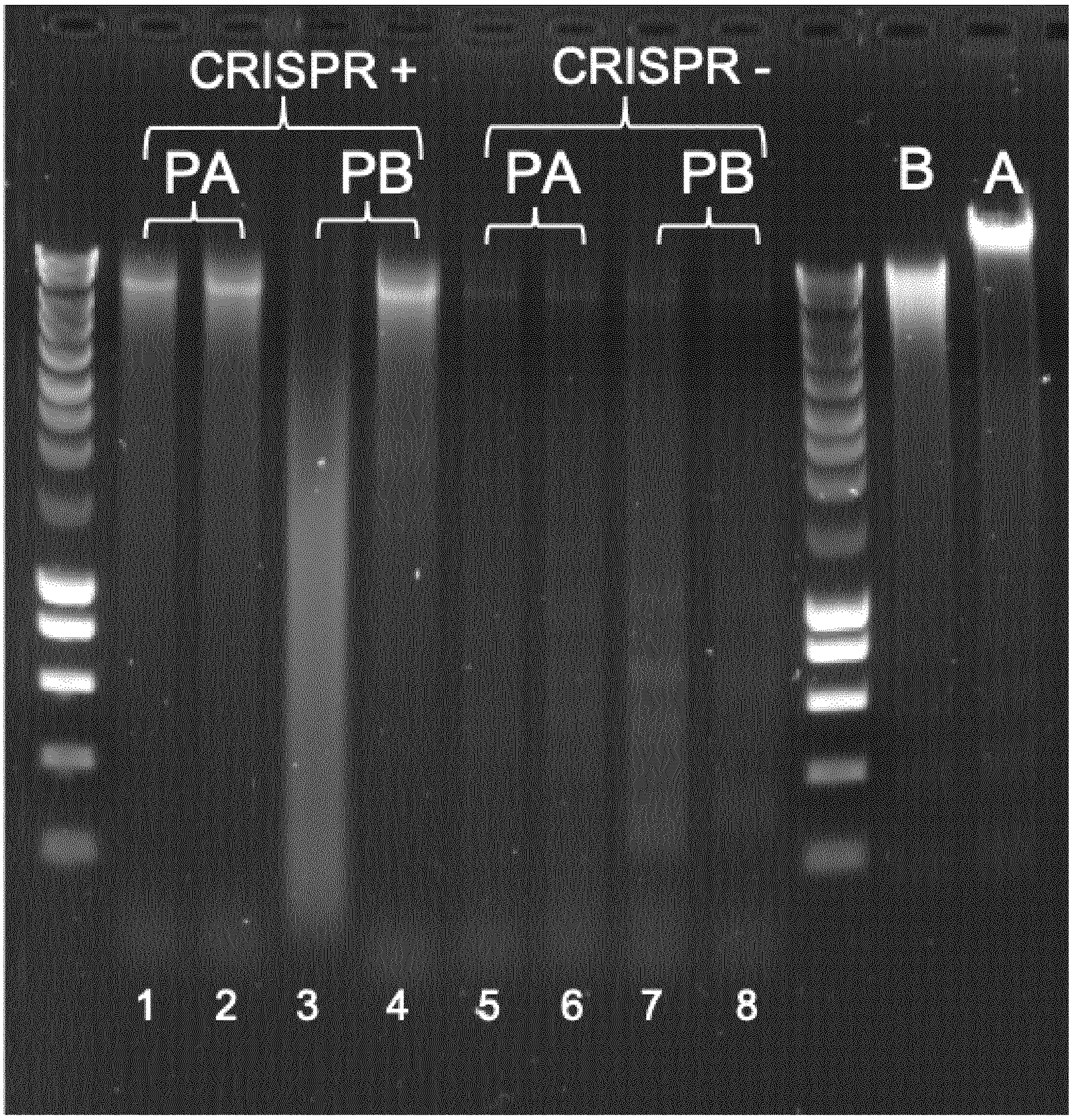
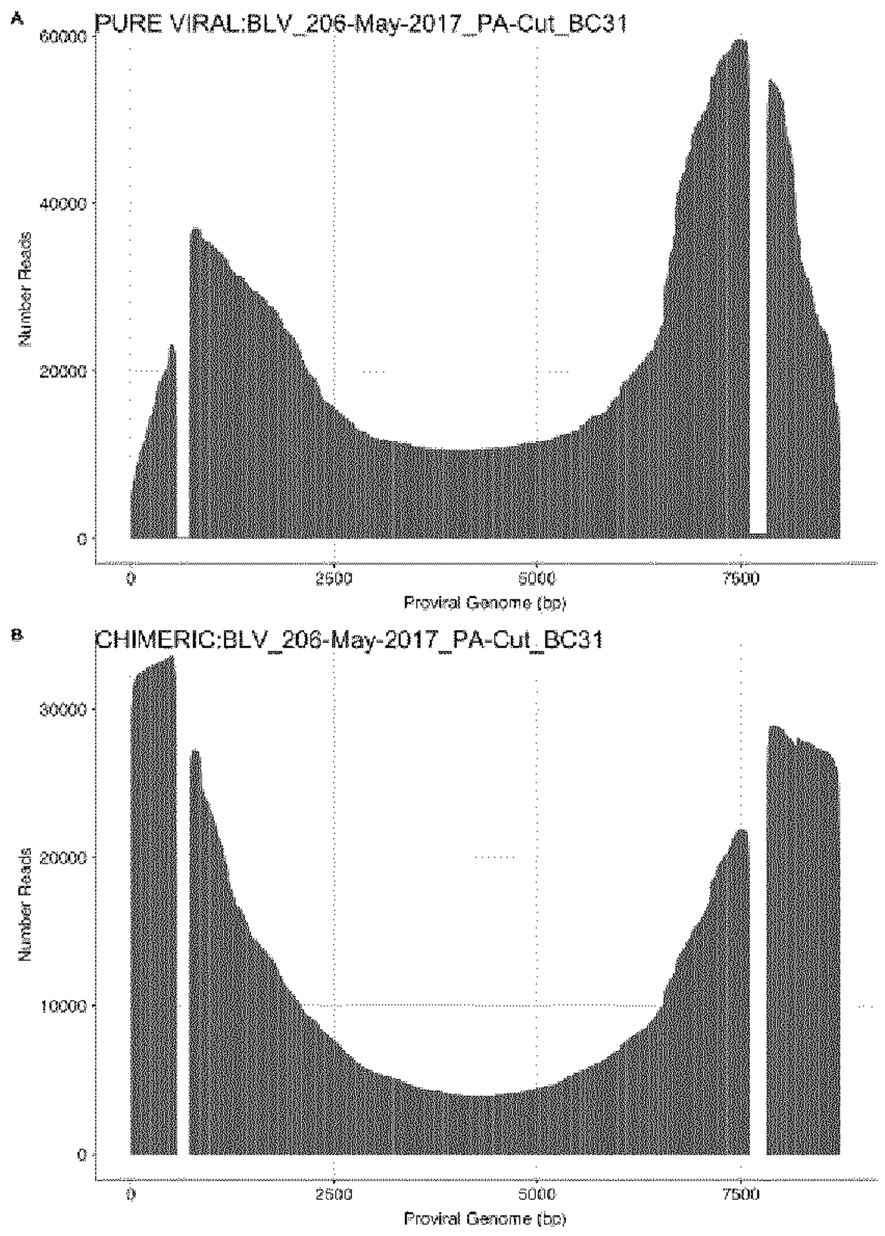
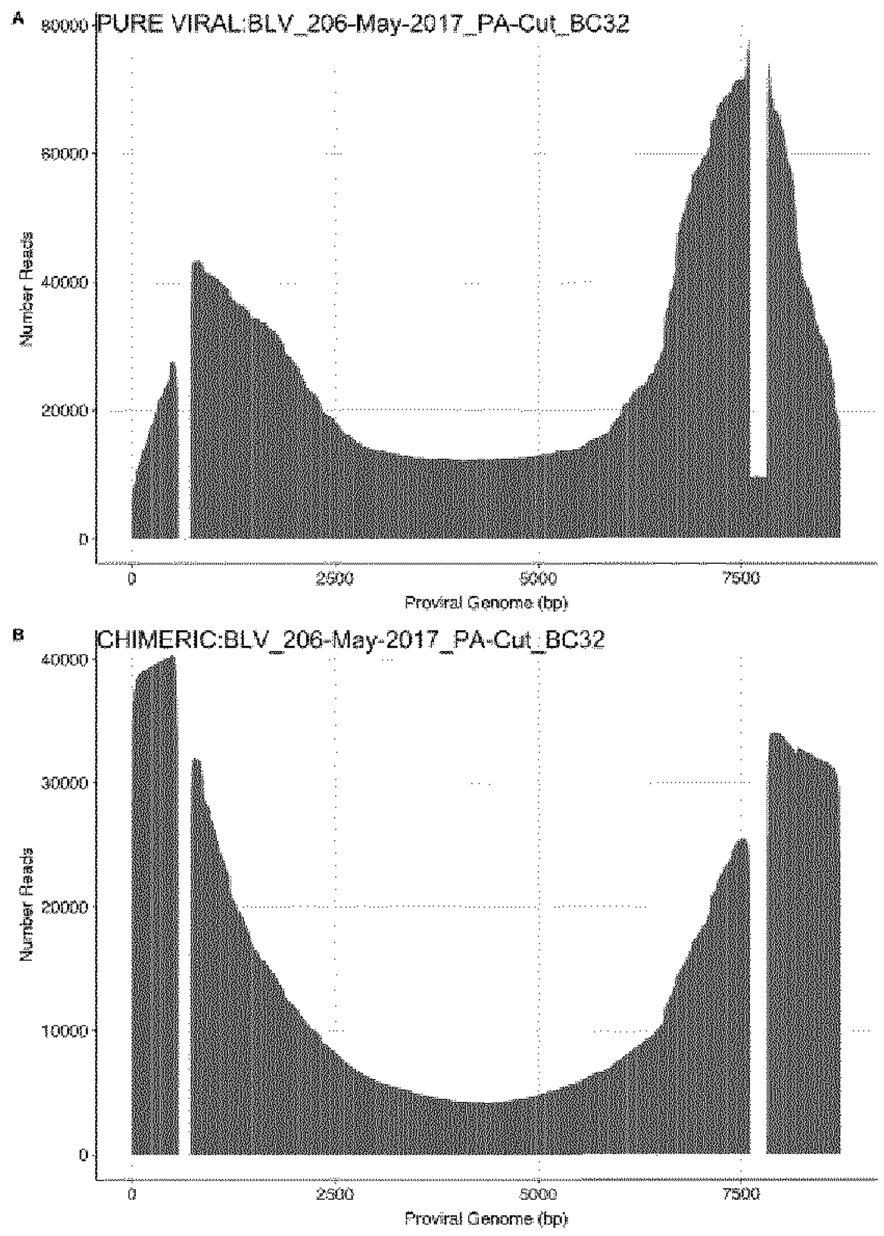
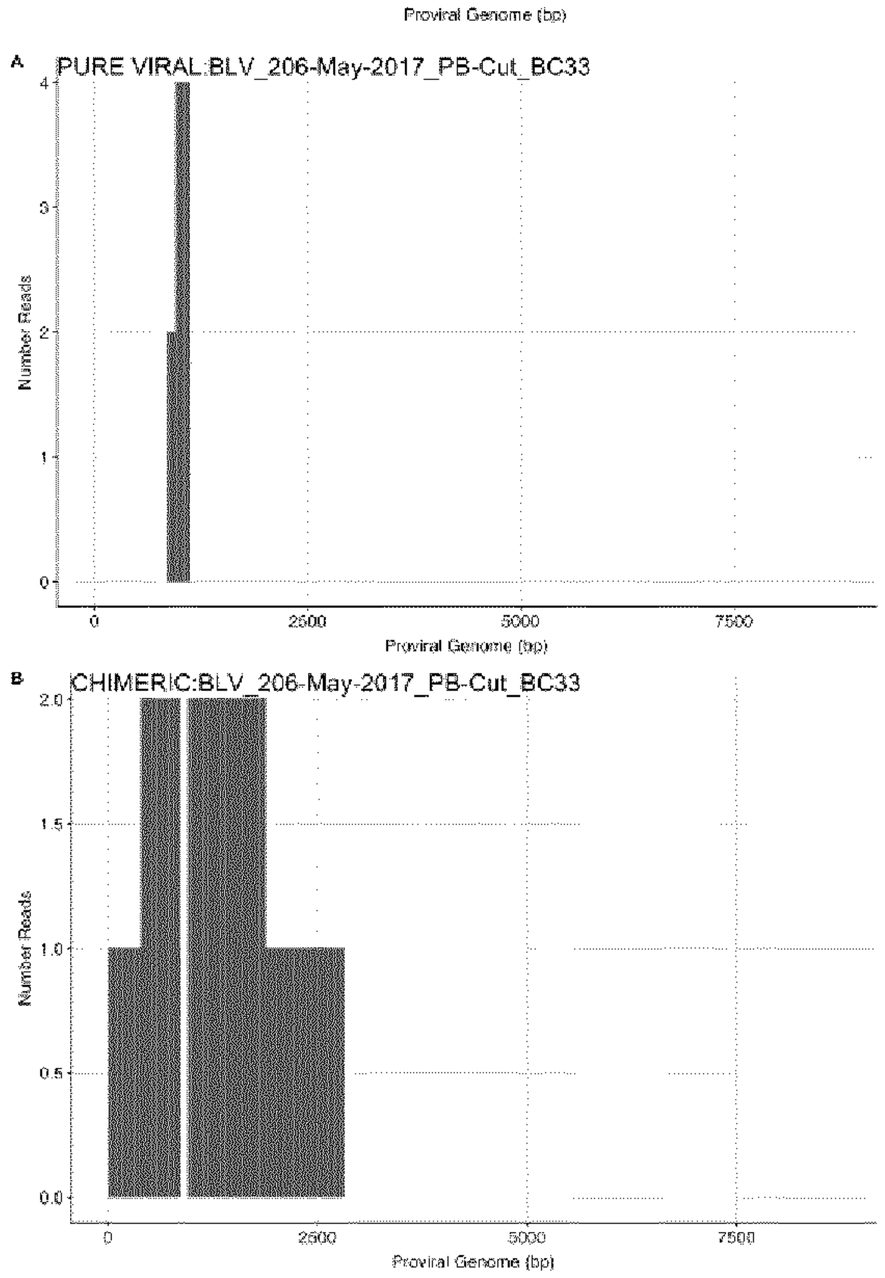


FIG. 9


A
PURE VIRAL:BLV_206-May-2017_PA-Cut_BC31
Number Reads
60000
40000
20000
0
0
2500
5000
7500
Proviral Genome (bp)
B
CHIMERIC:BLV_206-May-2017_PA-Cut_BC31
Number Reads
30000
20000
10000
0
0
2500
5000
7500
Proviral Genome (bp)


A
PURE VIRAL:BLV_206-May-2017_PA-Cut_BC32
Number Reads
80000
60000
40000
20000
0
0
2500
5000
7500
Proviral Genome (bp)
B
CHIMERIC:BLV_206-May-2017_PA-Cut_BC32
Number Reads
40000
30000
20000
10000
0
0
2500
5000
7500
Proviral Genome (bp)


A
PURE VIRAL:BLV_206-May-2017_PB-Cut_BC33
Number Reads
4
3
2
1
0
0
2500
5000
7500
Proviral Genome (bp)
B
CHIMERIC:BLV_206-May-2017_PB-Cut_BC33
Number Reads
2.0
1.5
1.0
0.5
0.0
0
2500
5000
7500
Proviral Genome (bp)

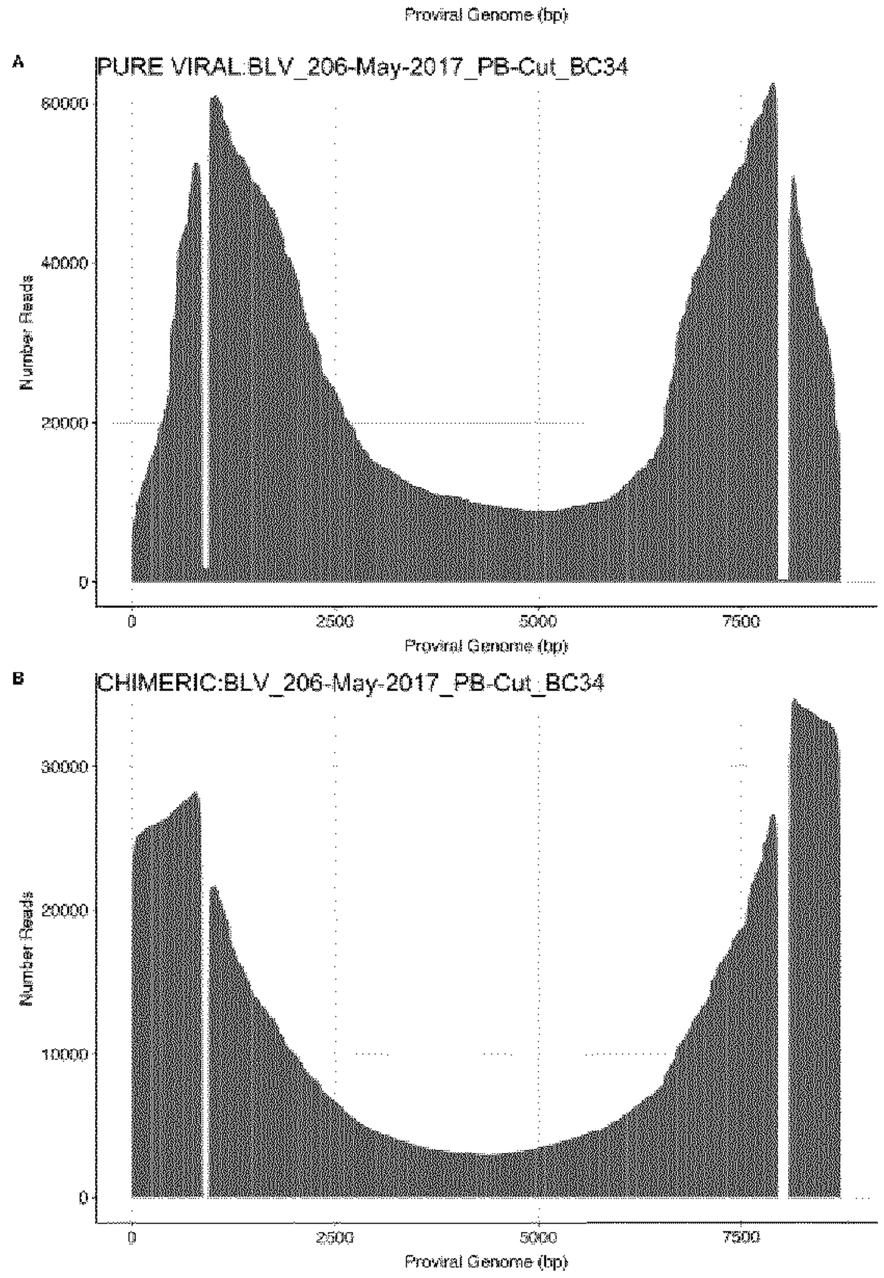
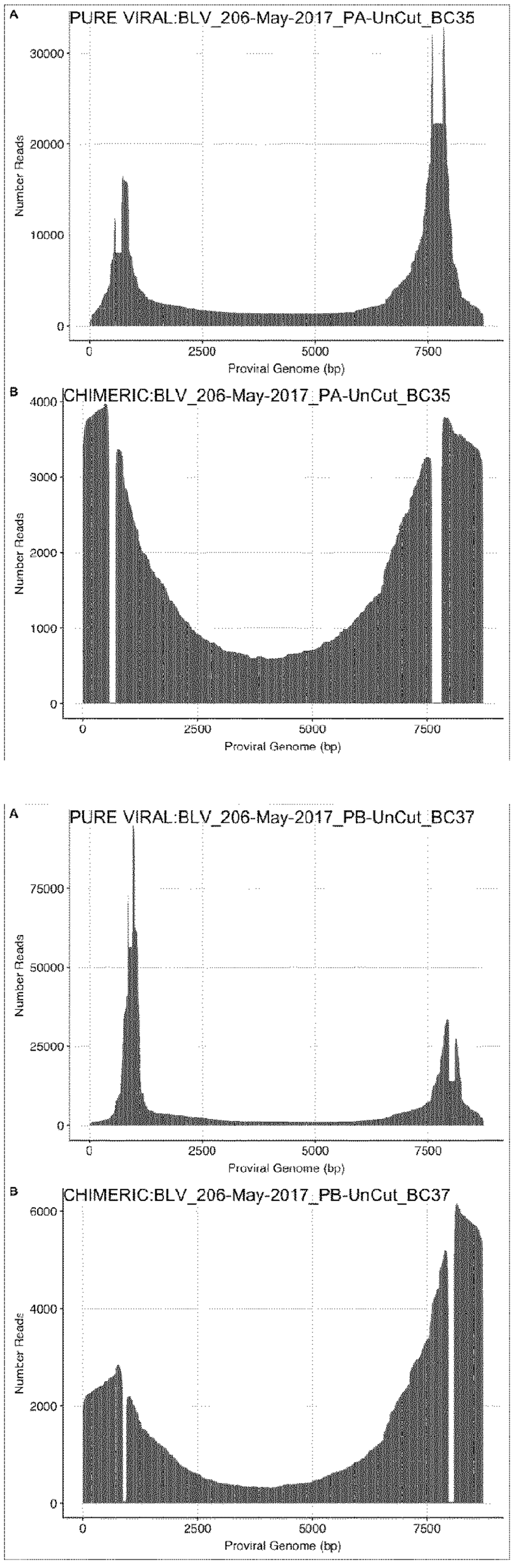

A
PURE VIRAL:BLV_206-May-2017_PB-Cut_BC34
Number Reads
60000
40000
20000
0
0
2500
5000
7500
Proviral Genome (bp)
B
CHIMERIC:BLV_206-May-2017_PB-Cut_BC34
Number Reads
30000
20000
10000
0
0
2500
5000
7500
Proviral Genome (bp)

FIG. 9 (cont.)


A
PURE VIRAL:BLV_206-May-2017_PA-UnCut_BC35
Number Reads
Proviral Genome (bp)
B
CHIMERIC:BLV_206-May-2017_PA-UnCut_BC35
Number Reads
Proviral Genome (bp)
A
PURE VIRAL:BLV_206-May-2017_PB-UnCut_BC37
Number Reads
Proviral Genome (bp)
B
CHIMERIC:BLV_206-May-2017_PB-UnCut_BC37
Number Reads
Proviral Genome (bp)

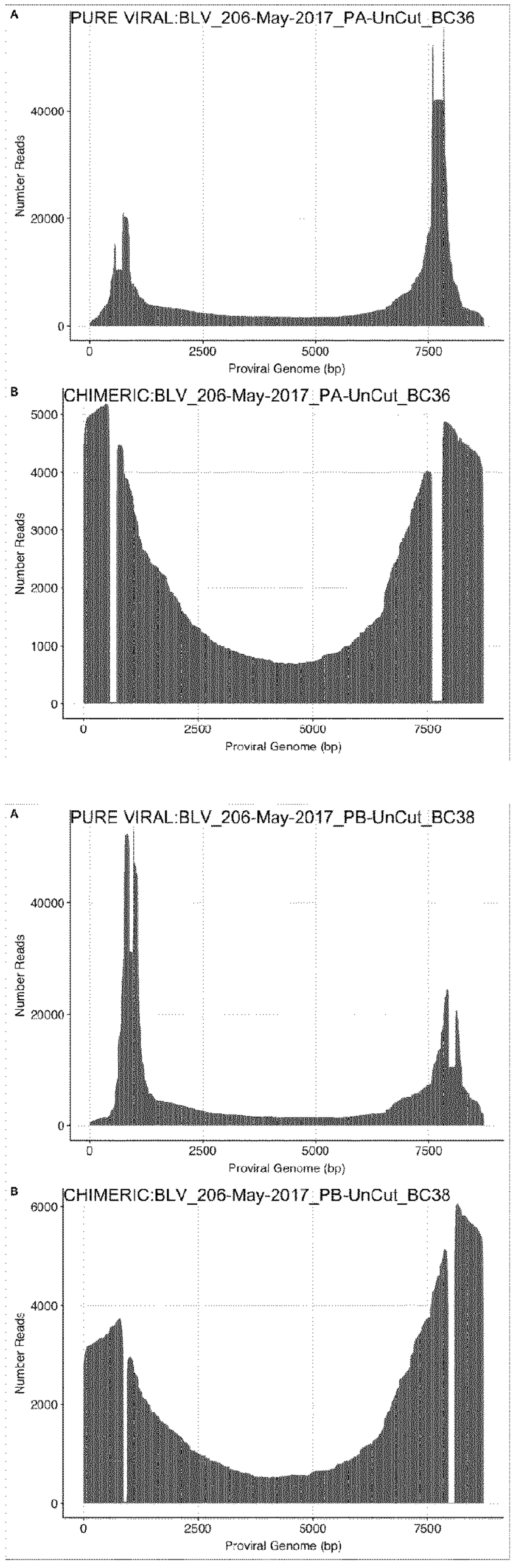
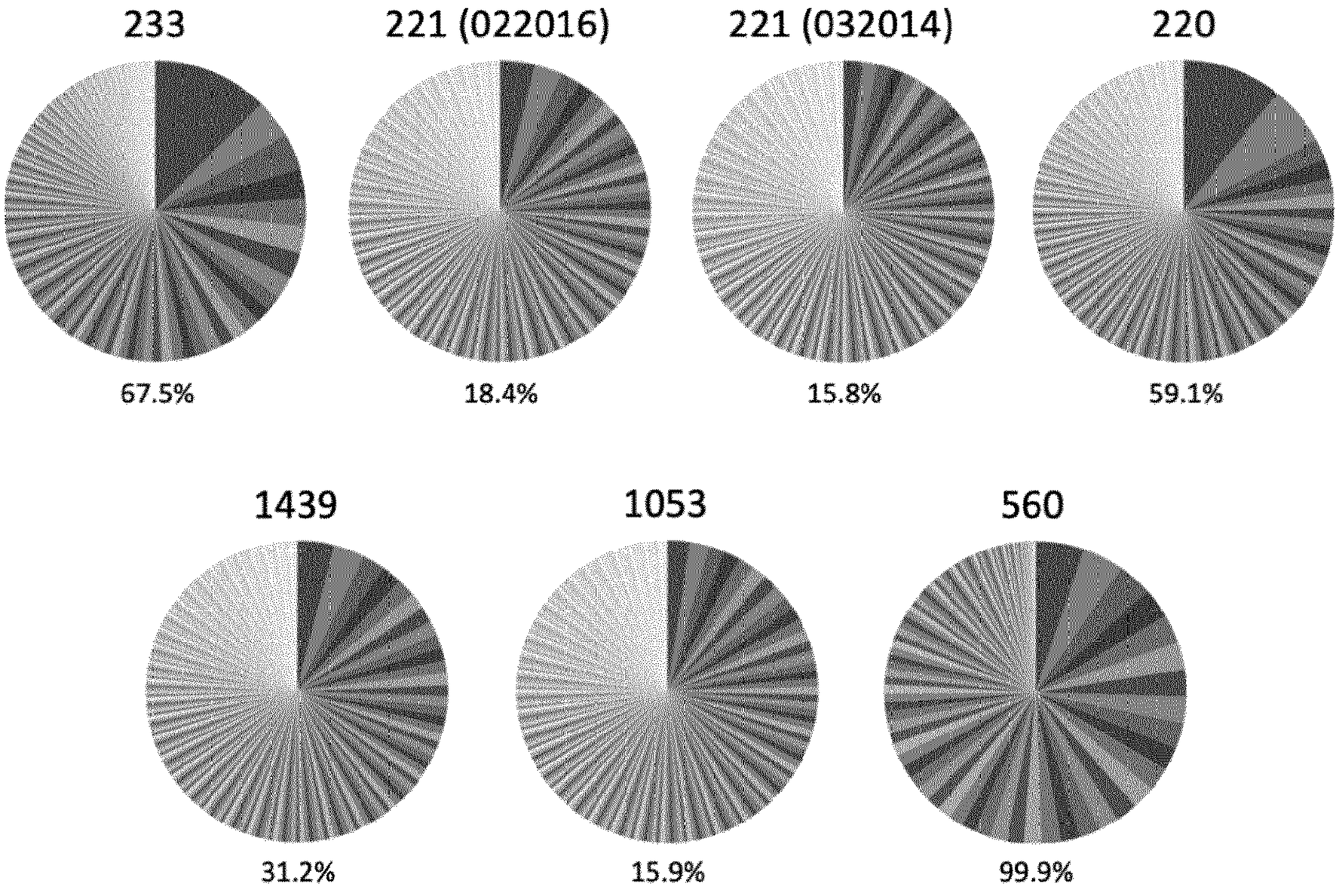

A
PURE VIRAL:BLV_206-May-2017_PA-UnCut_BC36
Number Reads
Proviral Genome (bp)
B
CHIMERIC:BLV_206-May-2017_PA-UnCut_BC36
Number Reads
Proviral Genome (bp)
A
PURE VIRAL:BLV_206-May-2017_PB-UnCut_BC38
Number Reads
Proviral Genome (bp)
B
CHIMERIC:BLV_206-May-2017_PB-UnCut_BC38
Number Reads
Proviral Genome (bp)

FIG. 10


233
67.5%
221 (022016)
18.4%
221 (032014)
15.8%
220
59.1%
1439
31.2%
1053
15.9%
560
99.9%

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/084557

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/6806 C12Q1/70 C12N15/113
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Artesi Maria ET AL: "Pooled CRISPR Inverse PCR sequencing (PCIP-seq): simultaneous sequencing of retroviral insertion points and the associated provirus in thousands of cells with long reads", bioRxiv, 24 February 2019 (2019-02-24), pages 1-23, XP055773318, DOI: 10.1101/558130 Retrieved from the Internet: URL:https://orbi.uliege.be/bitstream/2268/237583/1/558130.full%20(2).pdf [retrieved on 2021-02-08] the whole document abstract, p. 3, last para. - p. 4, first para.; Fig. 1 ----- -/--	1-20

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 February 2021	19/02/2021

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Sauer, Tincuta

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/084557

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 108 103 151 A (UNIV SOUTHEAST) 1 June 2018 (2018-06-01)	11-16
Y	the whole document SEQ ID NO: 20 and 24 on p. 26	1-10, 17-20

X	ZHEN SHUAI ET AL: "Synergistic antitumor effect on cervical cancer by rational combination of PD1 blockade and CRISPR-Cas9-mediated HPV knockout", CANCER GENE THERAPY, APPLETON & LANGE, GB, vol. 27, no. 3-4, 27 August 2019 (2019-08-27), pages 168-178, XP037096070, ISSN: 0929-1903, DOI: 10.1038/S41417-019-0131-9 [retrieved on 2019-08-27]	11-16
Y	the whole document abstract; p. 169, col. 2, p. 170, col. 1; p. 171, col. 2	1-10, 17-20

X	US 2019/201501 A1 (QUAKE STEPHEN R [US] ET AL) 4 July 2019 (2019-07-04)	11-16
Y	the whole document para. 3, 5, 7, 98, 149, 206, claims 22-26	1-10, 17-20

X,P	Artesi Maria ET AL: "Pooled CRISPR Inverse PCR sequencing (PCIP-seq): simultaneous sequencing ofretroviral insertion points and the integrated provirus with long reads", bioRxiv, 6 December 2019 (2019-12-06), pages 1-31, XP055773464, DOI: 10.1101/558130 Retrieved from the Internet: URL:https://www.biorxiv.org/content/10.1101/558130v3.full.pdf [retrieved on 2021-02-08]	1-20
	the whole document abstract, p. 19, Fig. 1	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2020/084557

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 108103151 A	01-06-2018	NONE	
US 2019201501 A1	04-07-2019	CA 3000155 A1	08-12-2016
		EP 3324999 A1	30-05-2018
		GB 2543873 A	03-05-2017
		JP 2018516984 A	28-06-2018
		US 2016346360 A1	01-12-2016
		US 2019201501 A1	04-07-2019
		WO 2016196282 A1	08-12-2016