P89 RNA-seq CAR transcript analysis

James Eddy

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Summary

I compared **coverage profiles** and **estimated abundances** for *CAR* and related transcripts using three sets of samples:

- **P89 bulk:** sorted CD8+ T-cells, sequenced as bulk populations (expected to express *CAR*)
- **P89 single-cell:** CD8+ T-cells, captured using **C1** instrument and sequenced invididually (expected to express *CAR*)
- **P85 single-cell:** MAIT-cells, captured using **C1** and sequenced individually (not expected to express *CAR*)

Based on read coverage across the *CAR* transcript, the construct appears to be (i) expressed in bulk samples from P89; (ii) not expressed in single-cell samples from P85; (iii) expressed in a fraction of single-cell samples from P89.

All code used for the pipeline and subsequent analysis (including this `Rmarkdown` report) is available at this repo: https://github.com/jaeddy/carTcellAnalysis

Pipeline overview

The *CAR* detection pipeline utilizes two tools: `Salmon` and `RapMap`. Both use the concept of "lightweight" / "quasi-" / "pseudo" alignment to rapidly map RNA-seq reads to the transcriptome. `Salmon` is primarily geared towards transcript *quantification*, using a probabilistic algorithm to estimate abundance (i.e., counts or TPM) for each reference transcript. `RapMap` provides a stand-alone version of the quasi-mapper used under the hood by `Salmon`. The output of `RapMap` is a SAM-like record of aligned reads, which enables further inspection of transcript coverage. These tools were chosen because they're extremely fast — ~2-5 minutes to map/quantify an RNA-seq library with ~5-10 million reads — allowing for rapid prototyping and testing.

For each library, reads are mapped to a modified human reference transcriptome, including all annotated transcripts from the hg38 gene model GTF **plus** the *CAR* transcript sequence.

Data used for analysis

Data presented below was generated and/or compiled from several sources:

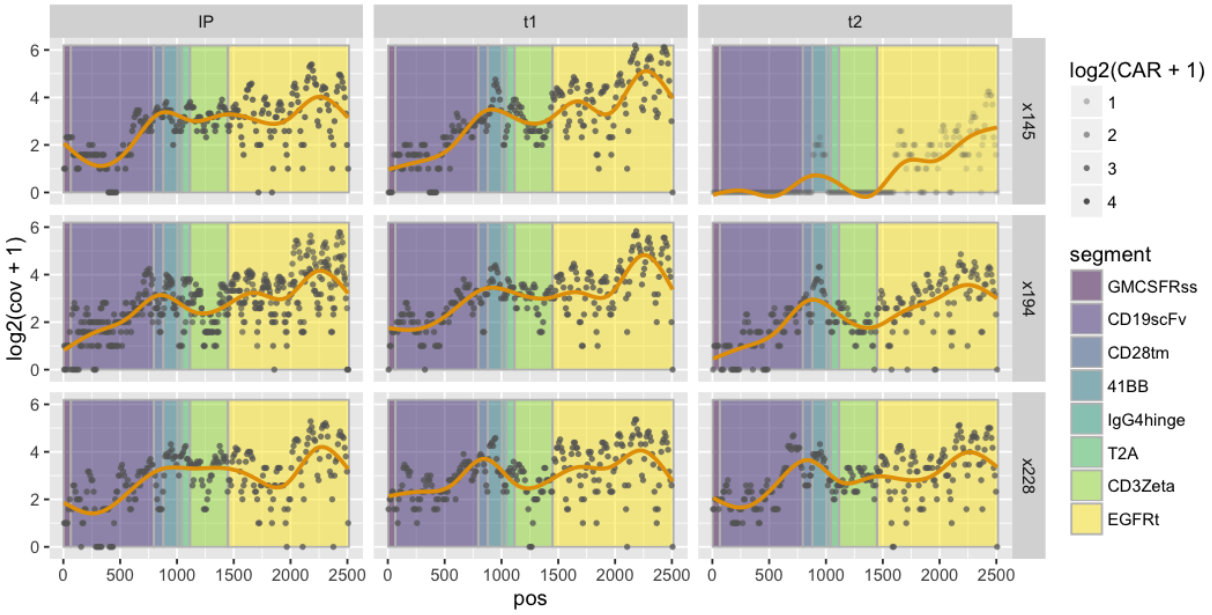
- **`sample_metrics_data`:** sample annotation (e.g., donor ID, timepoint, etc.) as well sequencing & alignment metrics from RNA-seq processing
- **`sample_rapmap_data`:** read coverage measured across the length of the *CAR* transcript and several *EGFR* transcript isoforms, based on mapping with the `RapMap` tool
- **`sample_salmon_data`:** abundance estimates (e.g., TPM, count) produced by the `Salmon` tool for *CAR* and several relevant transcripts when mapping to a modified human reference transcriptome (hg38)
- **`salmon_imgt_data`:** predicted/identified TCR junction sequences and alleles in single-cell libraries, as produced by assembly with `Trinity` followed by matching with `IMGT High V-QUEST`
- **`gff_file`:** custom-built GTF file describing where individual segments are located along the length of the *CAR* transcript

Results

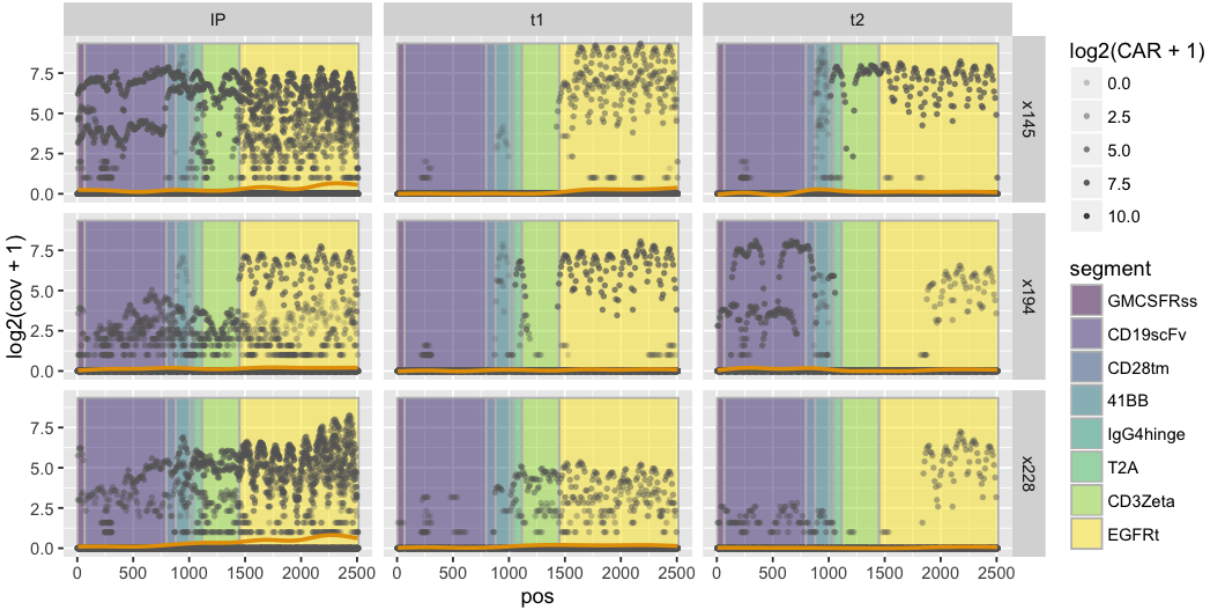
Visualizing *CAR* coverage & abundance

The plots below show read coverage from `RapMap` mapping across the length of the *CAR* sequence. Segments in the transcript, corresponding to the gene parts used to build the construct, are depicted by colored boxes in each plot. Transparency (i.e., alpha) is scaled based on the estimated abundance of the *CAR* transcript (TPM) as measured by `Salmon`.

Bulk libraries from project P89: While expressed at what would be considered low levels, the *CAR* transcript appears to be present in all donors at all timepoints. Note that for all but the **x194~IP** timepoint (2 replicates), each plot shows data from a single library.



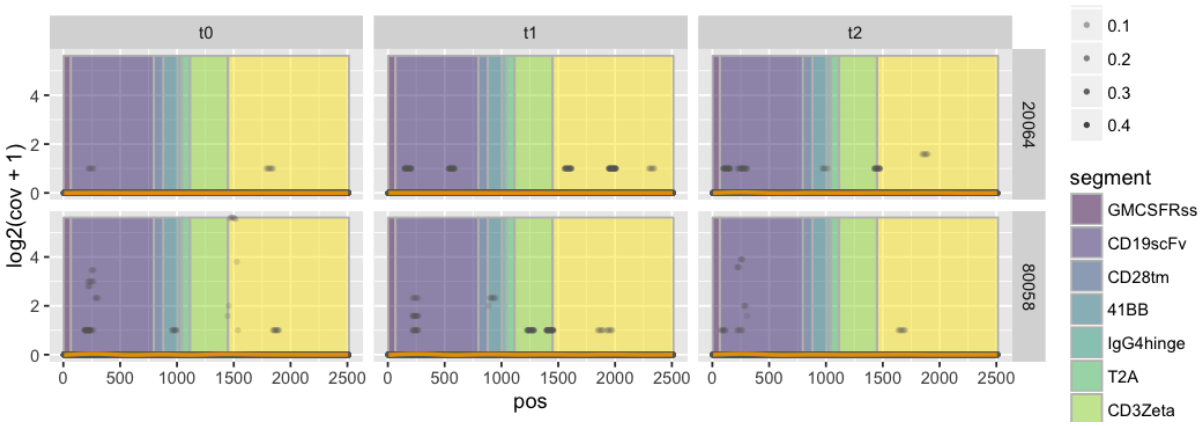
Single-cell libraries from project P89: In contrast, some single-cell libraries clearly show signs of *CAR* expression, but most do not. Each plot in this case shows data from all cells from a given donor/timepoint combination. The orange fit line depicts the average across all cells.



The table below shows the breakdown of libraries (cells) per group:

donor_id	timepoint	num_libs
x145	IP	79
x145	t1	56
x145	t2	60
x194	IP	71
x194	t1	74
x194	t2	57
x228	IP	58
x228	t1	35
x228	t2	69

Single-cell libraries from project P85: As expected for the negative control, the MAIT cells from P85 show virtually no evidence of *CAR* expression.

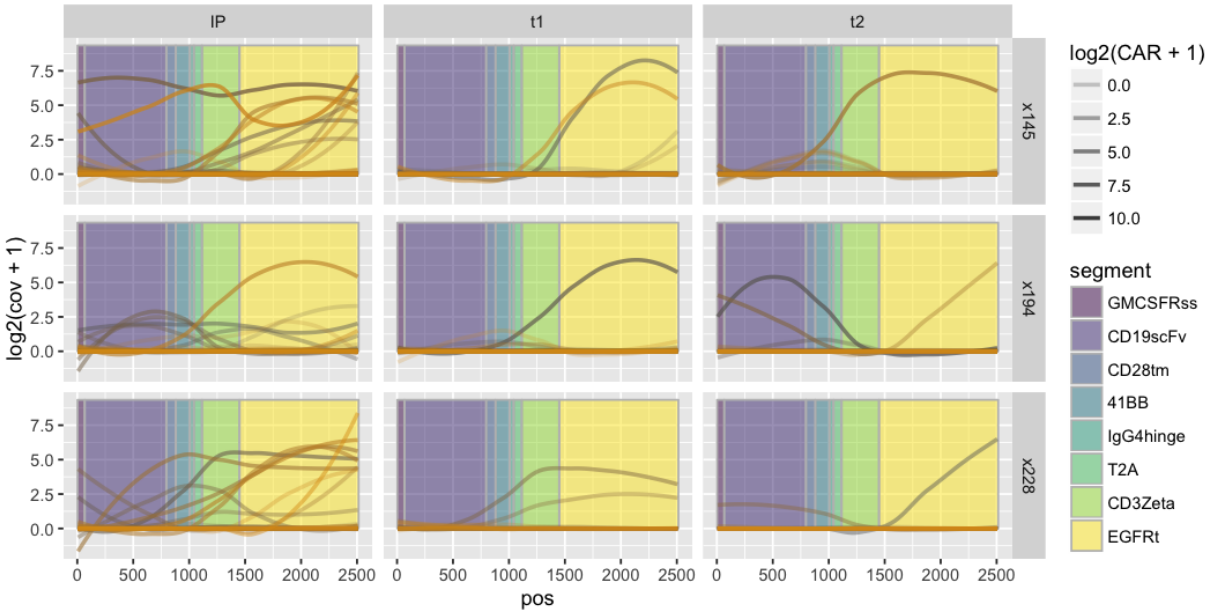


Here's the breakdown of libraries per group for P85:

donor_id	timepoint	num_libs
x145	IP	79
x145	t1	56
x145	t2	60
x194	IP	71
x194	t1	74
x194	t2	57
x228	IP	58
x228	t1	35
x228	t2	69

Inspecting trends in *CAR* coverage among single-cell P89 libs

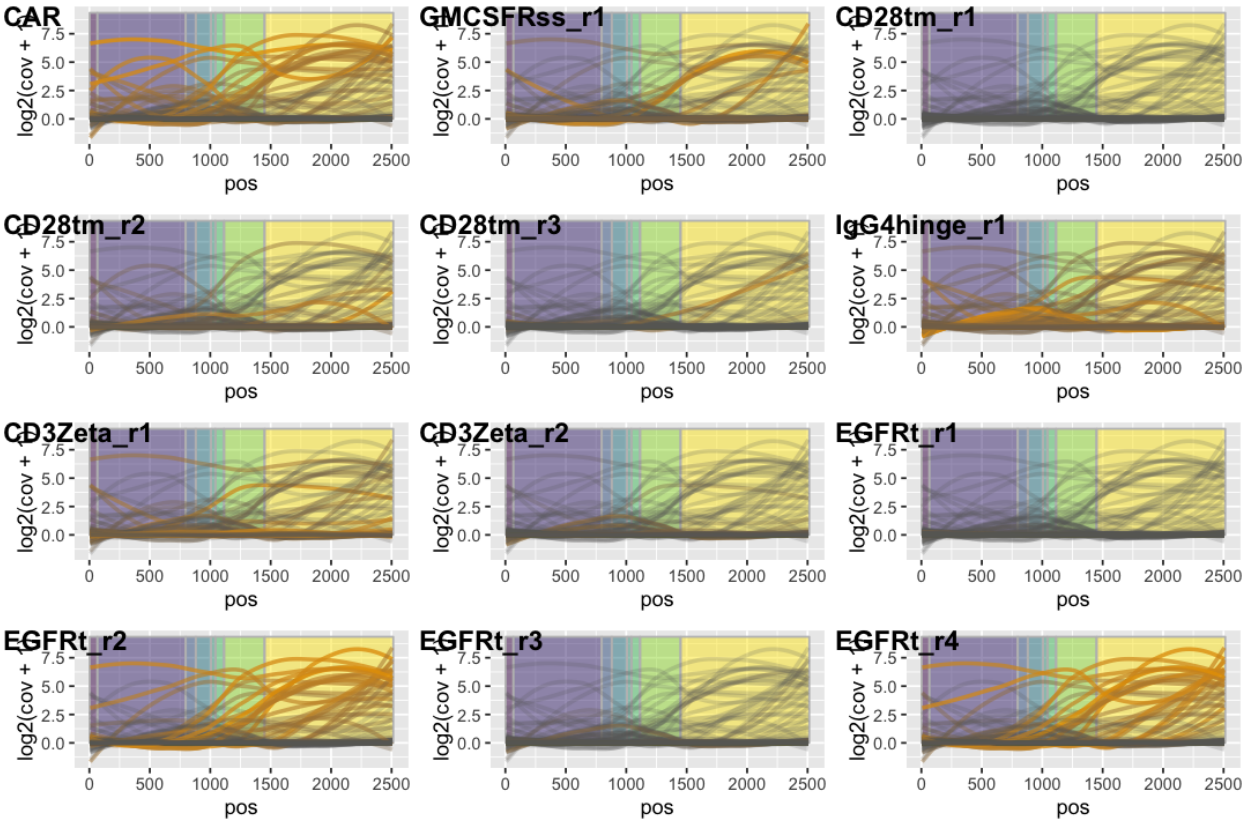
To simplify the plots (and make it easier to distinguish between libraries), below I've just shown the fit line of coverage for each library. Notably, these plots also show more clearly that, in many of the libraries with apparent *CAR* expression, the bulk of the signal appears to fall within the *EGFRt* region. I'll return to this point below.



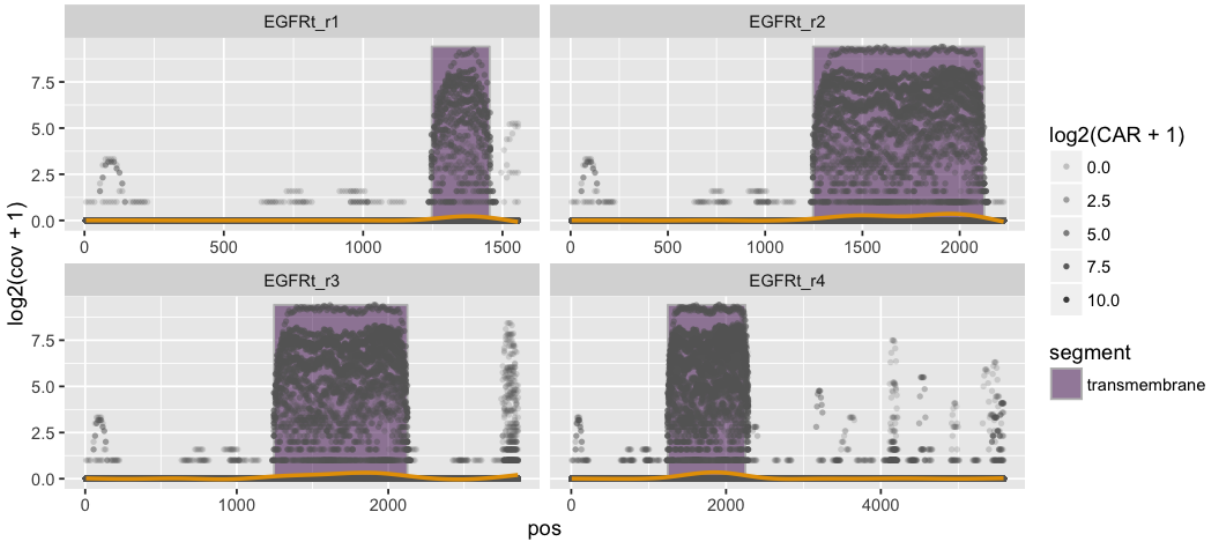
Correlation with abundance of non-*CAR* transcripts: The following human transcripts (which overlap the *CAR* sequence) were quantified by `Salmon`. The `xcript_name` (e.g., "CD28") corresponds to the "common" gene name for a particular *CAR* segment; `segment_version` includes a tag indicating record number, if multiple isoforms exist for the gene (e.g., "CD28tm_r1") in the reference transcriptome.

xcript_name	segment_version
CSF2	GMCSFRss_r1
CD28	CD28tm_r1
CD28	CD28tm_r2
CD28	CD28tm_r3
TNFRSF9	IgG4hinge_r1
CD247	CD3Zeta_r1
CD247	CD3Zeta_r2
EGFR	EGFRt_r1
EGFR	EGFRt_r2
EGFR	EGFRt_r3
EGFR	EGFRt_r4

Each plot shows *CAR* coverage across libraries, but colored based on the estimated abundance of the respective transcript. In other words, a "brighter" fit line in the plot for *EGFRt_r4* indicates higher expression of that transcript in a library, even though the lines themselves still represent *CAR* transcript coverage.



Estimated abundance of *EGFR* & *CAR* expression: Once again, higher estimated expression of some *EGFR* transcripts (particularly *EGFRt_r2* and *EGFRt_r4*) appears to correspond to stronger evidence of *CAR* expression. I took a closer look at this by identifying the subsequence of each transcript that overlaps with the *EGFRt* segment of the *CAR* gene, then plotting the coverage from `RapMap` for each transcript. The region matching *EGFRt* is highlighted in the purple box (i.e., "transmembrane").



It's pretty clear that the vast majority of reads mapping to any of the *EGFR* transcripts fall within the

transmembrane region. There are other subsequences with some coverage outside of the transmembrane region, but these are fairly short and may overlap with other transcripts in hg38.

As for **why** reads from the *CAR* transcript are mostly mapping to the *EGFRt* segment, there are at least a couple possible explanations:

1. Because the *CAR* transcript is fairly long, degradation from both the 5' and 3' ends could lead to less coverage on either end of the transcript;
2. Libraries prepared using the Nextera XT kit (or more specifically, using the Clontech SMARTer cDNA synthesis kit for C1) show a fair amount of 3' bias, which could further (or primarily) contribute to the skew observed along the *CAR* transcript.

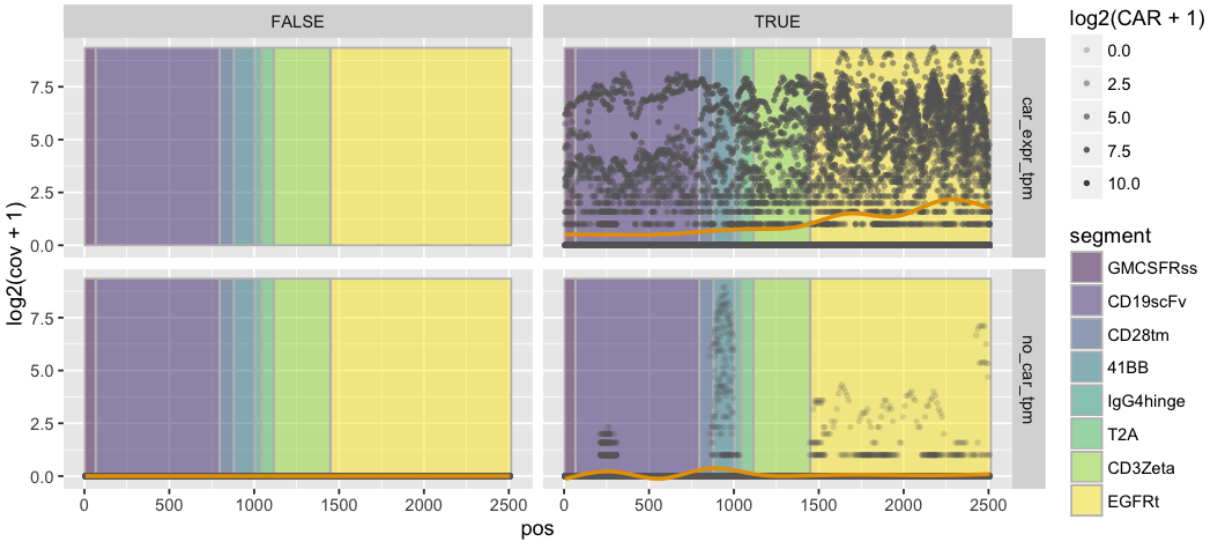
"Classifying" *CAR* detection in single-cell libraries

Based on the above observations, I tried to come up with a relatively simple way to classify whether *CAR* was detected in a particular library. In all cases, I set an absolute threshold for detection, based on whether a library had the bare minimum number of reads mapping to any position along the transcript. That is,

> non-zero coverage = TRUE if ≥ 5 positions with at least one read OR ≥ 1 position with at least two reads

Cases where a *CAR* detection rule and the non-zero coverage rule both return `FALSE` are considered to be "true negatives", giving some measure of specificity. When *CAR* is detected in libraries **without** non-zero coverage, this suggests a false positive.

Binarized *CAR* expression: Using the most straightforward criteria — positive expression of the *CAR* transcript (i.e., log2(TPM + 1) > 0 for *CAR*, as estimated by `Salmon`) — I appeared to miss a number of libraries that showed coverage of *EGFRt* or one of the non-human *CAR* segments (i.e., *CD19scFv*, *T2A*).

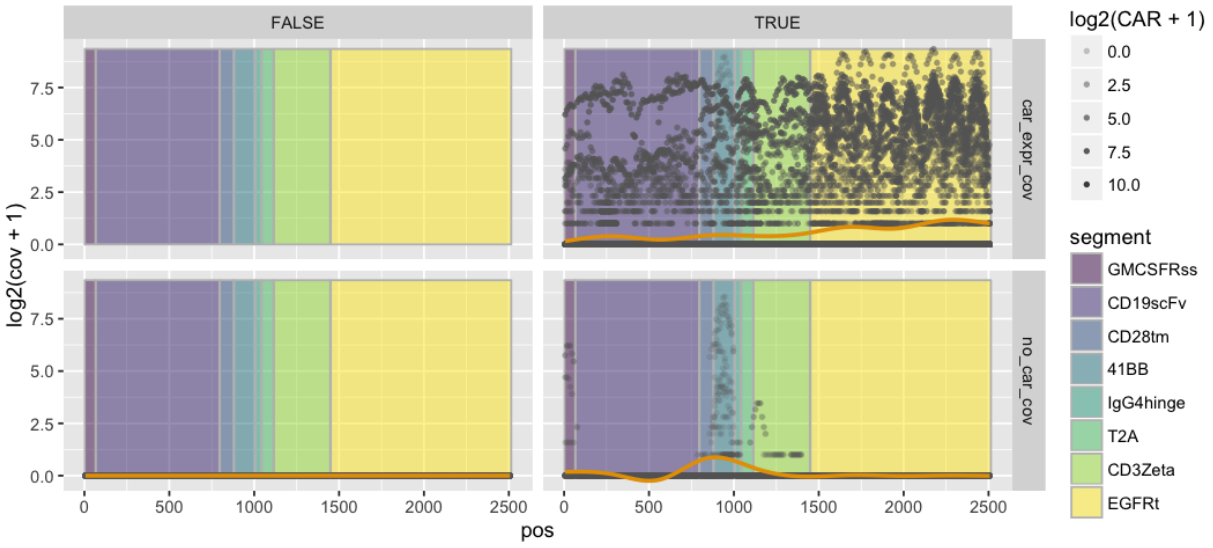


While there are no false positives in this case, and the specificity (true negative rate) appears solid, I would consider at least some of the libraries in the bottom right panel to be false negatives.

car_expr_tpm	nz_cov	n_libs
car_expr_tpm	TRUE	73
car_expr_tpm	NA	1
no_car_tpm	FALSE	391
no_car_tpm	TRUE	90
no_car_tpm	NA	4

Quantification-based rule: I tried to devise a more complicated rule based on the abundance of both the *CAR* transcript and each of the *EGFR* transcript isoforms (not shown). However, this lead to greater numbers of apparent false positives and false negatives, so I dropped this idea.

Coverage-based rule: Finally, putting together various pieces of information, I settled on a rule that checked for ≥ 2 *contiguous* (adjacent) positions with positive coverage in **ANY** of the segments *CD19scFV*, *T2A*, or *EGFRt*.

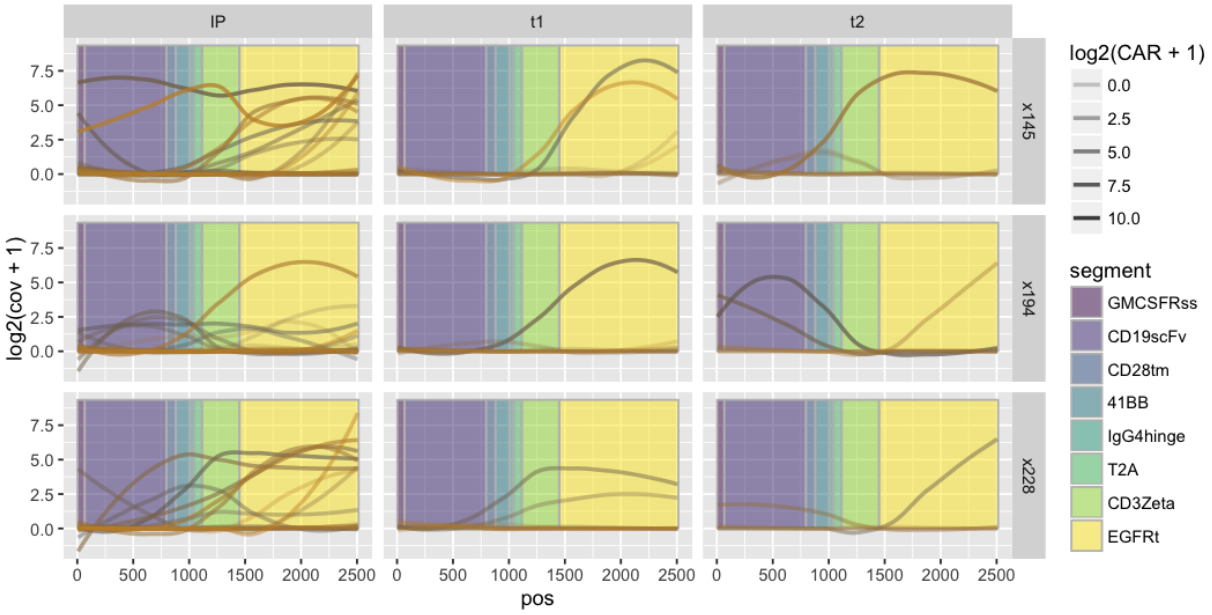


Based on coverage of these *CAR* "indicator" segments and the criteria of non-zero coverage across the *CAR* transcript, I would label 138 of the 559 single-cell libraries in P89 as "positive" for presence of the CAR transcript. Note: the pipeline produced an error for the `RapMap` step for 5 of the libraries, so I don't currently have coverage data for these; I'll need to go back and re-run this step.

car_expr_cov	nz_cov	n_libs
car_expr_cov	TRUE	138
no_car_cov	FALSE	391
no_car_cov	TRUE	25
NA	NA	5

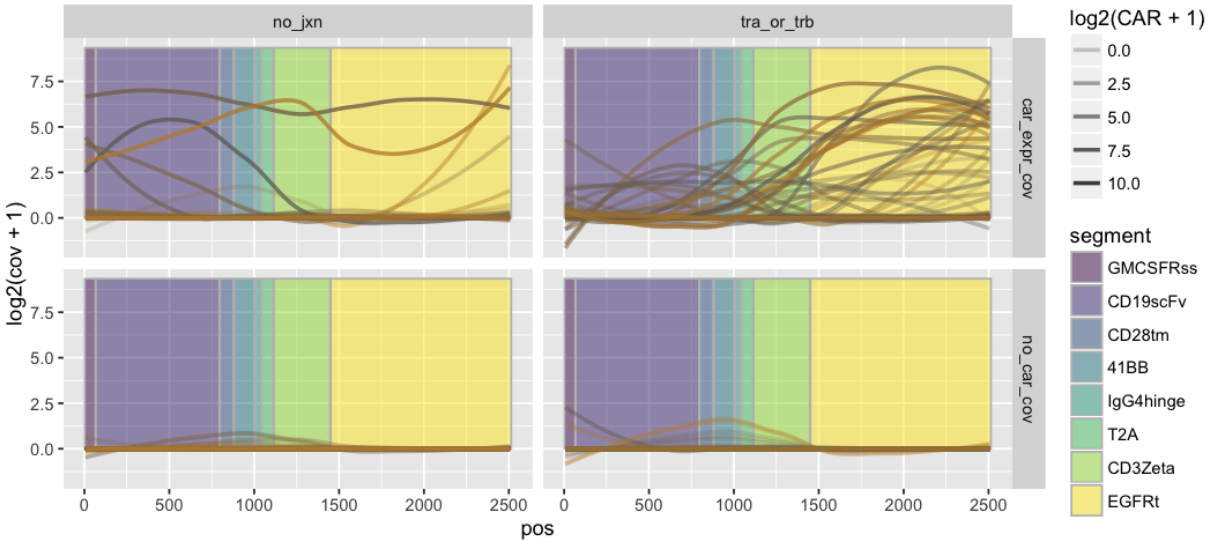
Revisiting *CAR* transcript trends across patients

With a more informed designation of *CAR* detection, below are plots of the remaining 138 libraries. Note that I haven't tried to control for library size in any way, so relative *abundances* (i.e., height on the y-axis) between libraries shouldn't be taken too seriously.



donor_id	timepoint	n_libs
x145	IP	29
x145	t1	13
x145	t2	9
x194	IP	28
x194	t1	12
x194	t2	7
x228	IP	24
x228	t1	11
x228	t2	5

As an additional check, I compared the overlap of libraries with detected *CAR* transcript and those for which we were able to identify either a functional *TRAV* or *TRBV* TCR junction. Not surprisingly, some libraries for which a TCR junction was identified did not appear to show evidence of *CAR* expression. On the other hand, TCR junctions were not recovered for several libraries with reasonably clear evidence of *CAR* coverage, suggesting that the junctions may have been missed due to limitation with sequencing parameters, transcriptome assembly tools, or junction identification tools.



Thoughts / next steps

1. I still don't have a great idea for how to *quantify* abundance of the *CAR* transcript in a particular library. I might be able to construct a reference transcript containing only the non-human segments and *EGFRt*, then map reads to **only** this transcript. On an absolute scale, I would expect the abundance estimates to be biased, but they might provide a reasonable indication of relative expression between libraries.

2. Beyond TCR junction detection, I haven't looked at correlation between *CAR* detection and any other features or phenotypes (including TCR clonotype). If there are any that seem like they might be especially interesting, that's something I could explore.

3. As far as key take-away points here, I would probably focus on 5 figures (we can discuss how to present those points in the most succinct way):

    i. *CAR* coverage in bulk P89 libraries;
    ii. *CAR* coverage in all single-cell P89 libraries;
    iii. *CAR* coverage in all single-cell P85 libraries;
    iv. *EGFR* coverage in single-cell P89 libraries;
    v. *CAR* coverage in those single-cell P89 libraries designated as *CAR*-positive

Detailed methods

Data preprocessing

Prior to any computation with `Salmon` and `RapMap`, several steps were needed to prepare reference data and indexes:

Formatting/building reference transcriptome To convert the *CAR* sequence information from lines in a Word document to something more usable (in this case, FASTA), I wrote a script to do most of the work for me. Writing the script took a bit of extra time, but it was useful for minimizing human error and replicated effort.

```
python scripts/format_fasta.py \
    data/sequence/carGeneRaw.txt \ # unformatted sequences, copied from Word doc into text file
    CAR \ # name of gene/transcript
    data/sequence/carTranscript.fasta \ # output FASTA file
    True \ # merge individual segments into a single FASTA record
    True # output is transcript (don't add artificial 'intron' buffer between segments)
```

To convert transcript records in the hg38 gene model GTF to the required FASTA format, I used the `gffread` function included with `cufflinks`.

```
gffread \
    -w data/sequence/hg38_transcripts.fa \ # output transcriptome FASTA
    -g genome.fa \ # reference genome (hg38) from iGenomes
    genes.gtf # reference gene models for hg38 from iGenomes
```

Finally, I simply pasted the *CAR* sequence to the top of the hg38 transcriptome FASTA.

```
cat data/sequence/carTranscript.fasta data/sequence/hg38_transcripts.fa > hg38_CAR_transcripts.fa
```

Formatting/building reference gene model GTF For the purposes of visualization and some filtering tasks, I created a pseudo-GTF file with a record for each segment of the *CAR* transcript. Each segment (e.g., "CD28tm") is labeled as a unique 'transcript', and the chromosome is denoted as *CAR-1*. The start and end position of each segment is also included. I was able to use the `format_fasta.py` script for this, keeping the default option to not merge segments.

```
python scripts/format_fasta.py \
    data/sequence/carGeneRaw.txt \ # unformatted sequences, copied from Word document into text file
    CAR \ # name of gene/transcript
    data/sequence/carGeneParts.fasta \ # output FASTA file
```

Using a bit of digging and manual editing, I also added entries for each transcript in the reference human transcriptome that might overlap with *CAR* segments. In this case, the transcript ID (e.g., "NM_001243077") is used as the chromosome name, the "common" gene name (e.g., "CD28") is used as the gene ID, and the corresponding *CAR* segment along with a tag indicating record number, if multiple isoforms exist for the gene (e.g., "CD28tm_r1").

```
python scripts/gene_fasta_to_gtf.py \
    data/sequence/carGeneParts.fasta \
    CAR \
    data/annotation/carGeneParts.gtf
```

Building indexes The commands for building indexes with `RapMap` and `Salmon`. Note: `docker` was used to run `RapMap`, as the tool is not currently available for Mac OS.

```
docker run --rm -v ${PWD}/data:/home/data jaeddy/rapmap:0.1.0-pre \ # loading docker image
    rapmap quasiindex \ # command to use rapmap executable & quasiindex module
    -t data/sequence/hg38_CAR_transcripts.fa \ # input reference transcriptome
    -i data/indexes/rapmap/hg38_CAR \ # index folder/prefix
    -k 19 #
```

```
tools/SalmonBeta-0.5.0_OSX-10.10/bin/salmon index # salmon executable & index module \
    -t data/sequence/hg38_CAR_transcripts.fa \
    -i data/indexes/salmon/hg38_CAR \
    --type quasi \
    -k 19
```

Mapping & quantification

Both index building and actual mapping/quantification are included in the script `car_detect_pipe.sh`, which runs the pipeline an all library FASTQ files specified in a tab-delimited input list. The `bash` code is a bit messy, so I won't include it here, but the `RapMap` step proceeds as follows:

1. Map reads to reference transcriptome, save output SAM file
2. Convert SAM to BAM, sort, and index with `samtools`
3. Filter BAM records to only include reads mapping to *CAR* or relevant endogeneous transcripts (using `samtools`)
4. Index again with `samtools`)

For the `Salmon` step, outputs are saved as-is.

Compiling & formatting outputs

I used several R scripts to read, parse, and format the outputs from `RapMap` and `Salmon`. In the interest of time, I haven't described what those scripts do here, but I can add that later.

Session info

```
## R version 3.2.1 (2015-06-18)
## Platform: x86_64-apple-darwin13.4.0 (64-bit)
## Running under: OS X 10.11.4 (unknown)
##
## locale:
## [1] en_US.UTF-8/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8
##
## attached base packages:
## [1] stats4    parallel  stats     graphics  grDevices utils     datasets
## [8] methods   base
##
## other attached packages:
##  [1] cowplot_0.6.1        scales_0.4.0         ggthemes_3.0.2
##  [4] rtracklayer_1.30.4   GenomicRanges_1.22.4 GenomeInfoDb_1.6.3
##  [7] IRanges_2.4.8        S4Vectors_0.8.11     BiocGenerics_0.16.1
## [10] viridis_0.3.4        ggplot2_2.1.0        dplyr_0.4.3
## [13] tidyr_0.4.1          stringr_1.0.0        knitr_1.12.3
##
## loaded via a namespace (and not attached):
##  [1] Rcpp_0.12.4                highr_0.5.1
##  [3] futile.logger_1.4.1        formatR_1.3
##  [5] plyr_1.8.3                 XVector_0.10.0
##  [7] futile.options_1.0.0       bitops_1.0-6
##  [9] tools_3.2.1                zlibbioc_1.16.0
## [11] digest_0.6.9               lattice_0.20-33
## [13] nlme_3.1-126               evaluate_0.8.3
## [15] gtable_0.2.0               mgcv_1.8-12
## [17] Matrix_1.2-4               DBI_0.3.1
## [19] yaml_2.1.13                gridExtra_2.2.1
## [21] Biostrings_2.38.4          grid_3.2.1
## [23] Biobase_2.30.0             R6_2.1.2
## [25] XML_3.98-1.4               BiocParallel_1.4.3
## [27] rmarkdown_0.9.5            reshape2_1.4.1
## [29] lambda.r_1.1.7             magrittr_1.5
## [31] codetools_0.2-14           GenomicAlignments_1.6.3
## [33] Rsamtools_1.22.0           htmltools_0.3.5
## [35] SummarizedExperiment_1.0.2 assertthat_0.1
## [37] colorspace_1.2-6           labeling_0.3
## [39] stringi_1.0-1              lazyeval_0.1.10
## [41] RCurl_1.95-4.8             munsell_0.4.3
```