

1 **Next-generation detection of antigen-responsive T cells using DNA barcode-labeled MHC-I**
2 **multimers**

3

4 Authors:

5 Amalie Kai Bentzen¹, Andrea Marion Marquard², Rikke Lyngaa¹, Sunil Kumar Saini¹, Sofie
6 Ramskov¹, Marco Donia³, Lina Such¹, Andrew J.S. Furness^{4,5}, Nicholas McGranahan^{4,6}, Rachel
7 Rosenthal^{4,6}, Per thor Straten³, Zoltan Szallasi², Inge Marie Svane³, Charles Swanton^{4,6}, Sergio A.
8 Quezada^{4,5}, Søren Nyboe Jakobsen^{1,7}, Aron Charles Eklund², Sine Reker Hadrup^{1,*}

9

10 Affiliations:

11 ¹Section for Immunology and Vaccinology, National Veterinary Institute, Technical University of
12 Denmark, 1870 Frederiksberg C, Denmark

13 ²Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of
14 Denmark, 2800 Lyngby, Denmark

15 ³Center for Cancer Immune Therapy, Herlev University Hospital, 2730 Herlev, Denmark

16 ⁴CRUK Lung Cancer Center of Excellence, UCL Cancer Institute, London, UK.

17 ⁵Cancer Immunology Unit, UCL Cancer Institute, University College London, UK

18 ⁶Translational Cancer Therapeutics Laboratory, The Francis Crick Institute 44 Lincoln's Inn Fields,
19 London, UK.

20 ⁷Immudex, 2100 Copenhagen, Denmark

21 *Correspondence: sirha@vet.dtu.dk (S.R.H.)

22

23 Contact Information:

24 Sine Reker Hadrup, Technical University of Denmark, Bülowsvej 27, 1870 Frederiksberg C,

25 Denmark

26

27 **ABSTRACT**

28 The identification of specific peptides recognized by T cells is important for understanding and
29 treating immune-related diseases. Current cytometry-based approaches are limited to the
30 simultaneous screening of T cell reactivity towards 10–100 distinct peptide specificities in a single
31 sample. We present a novel technology validated to screen for T cell recognition of multiple
32 (>1000) peptide specificities in a single sample using peptide-MHC multimers labeled with
33 individual DNA barcodes. Among MHC multimer-binding T cells the relative frequency of
34 sequenced DNA barcodes originating from a given peptide-MHC motif is related to the size of the
35 antigen-responsive T cell population. We demonstrate the application of DNA barcode-labeled
36 MHC multimers for the detection of rare T cell populations of both virus- and cancer-restricted
37 origins in various tissues. This technology enables true high-throughput detection of antigen-
38 responsive T cells and opens the possibility of genome-wide immune assessments on a personalized
39 basis.

40

41 **Main text**

42 CD8 T cells recognize peptide antigens presented by their cognate major histocompatibility
43 complex I (MHC I) molecule. This is a key element for adaptive immunity in the control of
44 intracellular pathogens, recognition and elimination of cancer and the pathogenesis of autoimmune
45 diseases. To understand disease development and to foster specific therapeutic interventions, it is

46 crucial to characterize the specific peptide-MHC molecules (pMHC) recognized by CD8 T cells of
47 relevance in a given disease. For the past two decades, antigen-specific T cells have been detected
48 using fluorescently labeled pMHC multimers¹. Currently, MHC multimer-based detection of
49 antigen-responsive T cells is limited by the number of fluorescent or metal tags available for either
50 flow- or mass cytometry^{2,3}. Combinatorial encoding, applying a specific combination of several tags
51 to a given pMHC multimer, has been employed to enhance the complexity of both analyses, which
52 has recently enabled the parallel screening of 120 different pMHC-responsive T cells in single
53 samples⁴⁻⁷. Although the combinatorial encoding principle has significantly enhanced our ability to
54 describe immune interactions, it is dwarfed by the vast number of potentially presented peptides and
55 the diversity of the T cell clones that recognize them^{8,9}.

56 DNA barcodes holds advantage as a novel type of tag for MHC multimers since oligonucleotides of
57 suitable lengths can be assembled to generate $>10^8$ different DNA barcodes¹⁰ and because the
58 composition of DNA barcodes in a sample can easily be determined through high-throughput
59 sequencing.

60 We provide proof-of-concept for the mixing >1000 different pMHC multimers tagged with unique
61 DNA barcodes to enable the parallel detection of potentially >1000 different pMHC-responsive T
62 cells in a single sample. The increased complexity of T cell detection enabled by use of DNA-
63 barcoded MHC multimers advances our understanding of immune recognition, from model antigens
64 to complex screenings that allow selection of candidate peptides on a genome-wide level, such as
65 the mutagenome of human cancer.

66

67 **RESULTS**

68 **Multi-parallel detection of antigen-responsive T cells in single samples**

69 To allow complex assessments of T cell reactivity in limited biological samples, we developed a
70 technology using DNA barcodes as tags for specific interactions between pMHC molecules and
71 CD8 T cells. DNA barcoded MHC multimer reagents also carrying a common fluorescent label,
72 phycoerythrin (PE), were generated on a dextran backbone as shown in Fig. 1a. This strategy
73 enabled single-tube-based detection of pMHC responsive T cells using mixtures of >1000 distinct
74 pMHC multimers where each specific pMHC molecule was associated with a given DNA barcode.
75 MHC multimer-binding T cells were isolated using fluorescence-activated cell sorting (FACS)
76 based on their PE fluorescence intensity, and the composition of the associated DNA barcodes were
77 identified through amplification and high-throughput sequencing. Antigen-responsive T cells within
78 the isolated T cell pool were identified based on the number of reads for a specific pMHC-
79 associated barcode compared to the complete pMHC multimer library. The number of unique DNA
80 sequences originating from each pMHC-associated barcode was assessed based on the
81 incorporation of unique molecular identifiers (UMIs)¹¹, allowing the clonal reduction of sequences
82 from the amplified product. This revealed the number of specific pMHC multimers that interacted
83 with T cells in the given cell sample.

84 The DNA barcodes were designed from sets of unique 25mer oligonucleotides using previously
85 published sequences described as having similar amplification properties while containing
86 maximum diversity of their identification motifs¹⁰. To provide a system adaptable to large library
87 screenings, we applied a combinatorial design of DNA barcodes as depicted in Fig. 1b and listed in
88 Supplementary Table 1-3.

89

90 **Feasibility and limit of detection using large libraries of DNA-barcoded MHC multimers for**
91 **T cell analyses**

92 To provide proof-of-feasibility for staining antigen-specific T cells in mixtures of >1000 different
93 pMHC multimers, we compared detection of various T cell populations responsive to virus-derived
94 peptides using DNA-barcoded MHC multimers or combinatorial fluorescently-labeled MHC
95 multimers, respectively^{4,12}. We verified that PE labeled MHC multimers carrying DNA barcodes
96 were able to bind specifically to their target T cell population even in excess of 999 irrelevant
97 pMHC multimers, and that the DNA barcode associated with positive control reagents could be
98 specifically recovered after isolation of MHC multimer binding cells (Supplementary Fig. 1a-d).

99 To investigate the linearity of detection and the detection limit of the method we prepared a titration
100 curve of antigen-specific T cells by mixing peripheral blood mononuclear cells (PBMCs) from one
101 donor (BC260) who carried ~5% HLA-B0702_CMV_pp65_{TPR}-specific T cells into an HLA-B0702-
102 negative donor (BC262) (Fig. 2a). T cell recognition of a panel of 1031 pMHC molecules
103 (Supplementary Table 4), each individually multimerized and encoded with a specific DNA
104 barcode, was assessed. Peptides were divided into different categories based on the origin of their
105 antigen. These categories included virus-derived antigens, melanoma-associated antigens¹³, Merkel
106 cell polyomavirus-derived antigens¹⁴, renal cell carcinoma-associated antigens and breast cancer-
107 associated antigens. Very few responses were detected, except within the virus-derived category
108 (Fig. 2b and Supplementary Fig. 2A). Here, we observed T cell responses to three epitopes in the
109 ‘5% sample’ (100% of BC260 PBMCs), i.e., HLA-B*0702 CMV_{TPR} (4.6% of CD8 T cells), HLA-
110 A*0201 CMV_{NLV} (0.15% of CD8 T cells), and HLA-A*0201 EBV_{YVL} (0.06% of CD8 T cells). In
111 the ‘1% sample’ (20% of BC260 PBMCs and 80% of BC262 PBMCs), we observed T cell
112 reactivity toward HLA-A*1101 EBV_{AVF} (0.1% of CD8 T cells) and HLA-A*0201 EBV_{GLC} (0.04%
113 of CD8 T cells) originating from BC262. Although the two low-frequency BC260-associated
114 responses, HLA-A*0201 CMV_{NLV} and EBV_{YVL} disappeared, the HLA-B*0702 CMV_{TPR} response
115 remained detectable. Stepwise 5-fold titrations led to a corresponding reduction in the detection of

116 the HLA-B*0702 CMV_{TPR}-associated DNA barcode and a loss of significance in terms of
117 sequence-read numbers at 0.008% of CD8 T cells, corresponding to an average of 20 MHC
118 multimer-positive T cells in a flow cytometry-based assessment (Fig. 2a-c). To compare T cell
119 detection based on DNA barcode-labeled MHC multimers to state-of-the-art flow cytometry based
120 assessment of pMHC specific T cell frequencies, the number of clonally reduced barcode reads was
121 applied to estimate the frequency of antigen-responsive T cells, based on an average number of
122 TCR-MHC multimer interactions in the total MHC multimer-binding T cell pool in a given cell
123 sample. This estimate was obtained from clonally reduced barcode reads retrieved from a given
124 sample and the frequency of all MHC multimer-positive T cells in that sample using the following
125 equation: (number of DNA barcode reads associated with a specific pMHC/number of total barcode
126 reads derived from the same sample) x percentage of MHC multimer-binding cells among total
127 CD8 T cells (Supplementary Table 5). The estimated frequencies of pMHC-responsive T cells
128 correlated strongly with the frequencies determined by combinatorial fluorescently-labeled MHC
129 multimers (Fig. 2d). When the HLA-B*0702 CMV_{TPR}-responsive T cells were assessed in an
130 identical set of samples using a smaller library of MHC multimers, n=110 (Supplementary Table 6),
131 we observed comparable detection limit (0.001% of CD8 T cells) and strong correlation with
132 detection via fluorescently labeled MHC multimers ($r^2=0.999$) (Supplementary Fig. 3a-d).
133 Moreover, strong correlations were observed when results from the 1031 MHC multimer library
134 were compared with the 110 MHC multimer library, both related to the estimated frequencies of
135 HLA-B*0702 CMV_{TPR}-specific T cells ($r^2=0.999$) and the clonally reduced read counts ($r^2=0.805$),
136 respectively (Supplementary Fig. 3d-e). The number of cells used for analysis could be increased at
137 least to 10×10^6 cells per sample while maintaining specific MHC multimer staining. Such 5-fold
138 increase in the number a precursor cells seems to increase the limit of detection (Fig. 2e).

139

140 **Detection of pMHC-responsive T cells across different donors**

141 We continued using the 1031 member MHC multimer library to screen for T cell reactivity in
142 PBMCs across 10 different healthy donors with various HLA types (Supplementary Table 7). We
143 observed a T cell response signature dominated by reactivity toward virus-derived peptide
144 sequences (Fig. 3a). Focusing on the virus-derived peptides, we observed a highly HLA-dependent
145 signature of T cell reactivity (Fig. 3b). BC171 expressed HLA-A*1101, A*0301, B*0702, and
146 B*1501, and the detected T cell reactivity corresponded to these tissue types. BC260, 259, 261,
147 261, 268, 254, and 251 expressed HLA-A*0201, and they showed variable T cell reactivity towards
148 HLA-A*0201-restricted virus-derived epitopes. BC268 expressed HLA-A*0101 and HLA-B*0801
149 and showed both CMV- and FLU-specific reactivity restricted to these HLA molecules. Among the
150 10 healthy donors screened with 1031 different pMHC multimers, we identify only one response
151 not matching the HLA type of the given individual (Fig. 3b, HLA-B*0801, HSV pU79_{EGR}, BC251).
152 T cell reactivity against virus-derived peptides was determined by flow cytometry in parallel. The
153 frequencies of antigen-responsive T cells determined using the two methods correlated strongly,
154 $r^2=0.967$ (Fig. 3c). Of 42 potential responses, 88% were detected either by fluorescent labeled MHC
155 multimers or by DNA-barcoded MHC multimers. The five responses detected by flow cytometry
156 but not with DNA-barcoded MHC multimers, were all present at low frequencies in the analyzed
157 cell samples (<0.01 % of CD8 T cells), and four of these were just below the threshold of 0.1%
158 FDR in the DNA-barcode based analysis. The additional T cell specificities detected with DNA-
159 barcoded MHC multimers and not with fluorescent labeled MHC multimers is likely to represent
160 specific detection because they all match the HLA-expression and virus-response profiles of the
161 given donors.

162 When PBMCs from six of the same donors were analyzed using a library of 110 DNA-barcoded
163 MHC multimers (Supplementary Fig. 4 and Supplementary Table 6), we again observed strong

164 correlations when comparing T cell reactivity detected using DNA-barcoded MHC multimers and
165 that detected using combinatorial fluorescently labeled MHC multimers, $r^2=0.985$ (Supplementary
166 Fig. 4c), and when comparing results from the 110 versus the 1031 member pMHC library,
167 $r^2=0.951$ (Supplementary Fig. 4d-e). Thus, indicating that library size does not considerably affect
168 the capacity for detection of specific T cells using DNA-barcoded MHC-multimers.

169

170 **Detection of tumor-reactive T cells**

171 Recent clinical success in cancer immunotherapy has led to great interest in examining T cell
172 reactivity against cancer. To demonstrate the feasibility of using DNA-barcoded MHC multimers
173 for the detection of tumor-restricted pMHC-specific T cells, we analyzed T cell reactivity among
174 tumor-infiltrating lymphocytes (TILs) from melanoma patients against a previously described
175 library of shared melanoma-associated peptides^{13,15}. Antigen-specificity was assessed within 11
176 expanded TIL products against an HLA-A*0201-restricted DNA-barcoded pMHC multimer library
177 of 167 melanoma-associated peptides and 8 virus-derived peptides (Supplementary Table 8). We
178 detected numerous T cell populations responsive to melanoma associated antigens in 8 of the 11
179 TIL products (Fig. 4A-B). In comparison the same library of melanoma-associated epitopes was
180 included for T cell screening in the healthy donor cohort resulting in very few detected responses
181 (Fig. 3a). Interestingly, two patients not responding to these HLA-A*0201 presented peptides, were
182 evaluated as HLA-A*0205 positive upon detailed HLA typing (MM10 and MM11, Supplementary
183 Table 7), adding to previous observations that HLA micropolymorphisms may strongly affect T cell
184 detection using MHC multimers^{16,17}. T cell reactivity was detected against several well-known
185 melanoma-associated antigens, such as MART-1, NY-ESO-1, gp100 and tyrosinase. We observed
186 reactivity against all three variants of the MART-1 peptide (ELAGIGILTV, EAAGIGILTV, and
187 AAGIGILTV) within the same TIL samples in patients MM02 and MM03. This finding is likely to

188 reflect partial cross-recognition of all three peptides from the same T cell population, a phenomenon
189 that has been described previously¹⁸⁻²¹. Additionally, T cell reactivity against two different variants
190 of the gp100 peptide (ITDQVPFSV and IMDQVPFSV) and variants of the NY-ESO-1 peptide
191 (SLLMWITQC and SLLMWITQA) was detected in patient MM01 and MM02, respectively (Fig.
192 4b). Similar to our observations in the healthy donor cohort, there was a tight correlation between
193 the number of antigen-responsive T cells estimated based on association to DNA-barcoded MHC
194 multimers, and that detected using combinatorial fluorescently labeled MHC multimers, $r^2=0.901$
195 (Fig. 4c). In the melanoma samples, DNA barcode-based screening resulted in detection of an
196 increased numbers of melanoma-associated peptide-specific T cell populations (n=45) compared to
197 screening with fluorescently labeled MHC multimers (n=16). The additional peptide-restricted
198 populations detected by DNA-barcoded MHC multimers showed a patient-specific pattern that
199 matched the HLA expression and antigen-response signature of the patient (Fig. 4a-b) and the
200 detection of an increased number of tumor-specific T cell populations may reflect that T cell
201 detection based on DNA barcode-labeled MHC multimers provides less dependence on stringent
202 fluorescence-based separation (Supplementary Fig. 5a-b).

203 204 **Detection of tumor-reactive T cells from small-size clinical samples**

205 One major advantage of multiplex technologies for T cell detection is the possibility of determining
206 the composition of antigen-specific T cells in small biological samples without a need for
207 lymphocyte expansion. We utilized the high-throughput screening capacity of DNA-barcoded MHC
208 multimers to study the dynamics of T cell responses in various samples from two patients with
209 metastatic melanoma participating in a phase II trial, with adoptive cell therapy using in-vitro
210 expanded TILs²². We screened for T cell recognition towards a large library of virus- and shared
211 melanoma-derived epitopes (328 barcoded pMHC multimers, Supplementary Table 9) in: a)

212 uncultured tumor fragments following enzymatic digest, i.e. unexpanded TILs (digest), b) TILs in
213 vitro expanded from small tumor fragments, dissected from the same metastatic lesion and c)
214 peripheral blood obtained before and approximately 1 month after infusion of expanded TILs.
215 Although very few lymphocytes were available in the melanoma samples directly after enzymatic
216 digest (18,000 and 48,000, respectively for MM01 and MM02), we detected numerous melanoma-
217 associated T cell responses in these tissue samples (Fig. 5a-b). In MM01 two of these responses
218 were also detected in the expanded TILs and in peripheral blood after transfer (Fig. 5a). The
219 composition of T cell responses in peripheral blood after adoptive transfer was to a large extent
220 matching the composition of the TIL infusion product. In MM02, T cell responses detected in
221 peripheral blood after TIL transfer reflected the composition of the TIL infusion product, but also
222 additional responses were detected. These additional responses matched those detected in a digest
223 sample taken from a tumor lesion after tumor progression (Fig. 5b). Overall, we detected an
224 increased response to melanoma associated antigens in peripheral blood after adoptive transfer of
225 TILs, reflecting T cell populations detected in both tumor digest samples and expanded TIL
226 products.

227

228 **Detection of neoepitope-responsive T cells in non-small cell lung carcinoma**

229 Mutation-derived neoepitopes have been suggested as important targets for tumor rejection
230 mediated by T cells, and the predicted presence of neoepitopes correlates positively with clinical
231 outcome following treatment with immune checkpoint inhibitors^{23,24}. Consequently, efficient
232 means of identifying neoepitope-restricted T cells are of potential prognostic and therapeutic value.
233 We analyzed two non-small cell lung carcinoma (NSCLC) patients for T cell recognition of
234 predicted HLA-binding peptide sequences containing cancer-specific mutations, i.e., potential
235 neoepitopes. For each patient, a large personal neoepitope peptide library was identified, n=288 and

236 n=417 for patient L011 and L013, respectively (described in Supplementary Methods). T cell
237 reactivity of in vitro-expanded TILs was assessed using both combinatorial fluorescently labeled
238 MHC multimers and DNA-barcoded MHC multimers. The results from the first approach are
239 reported in detail in McGranahan et al, 2016²⁵, and resulted in detection of one neoepitope
240 responsive T cell population in L011. Using DNA-barcoded MHC multimers, we observed nine
241 neoepitope responsive T cell populations and one virus responsive T cell population in these two
242 patients (Fig. 6a-b). The use of DNA barcode-labeled MHC multimers allowed us to screen the
243 whole library in one tube (4-8 mill TILs) rather than using 10 parallel tubes (1 mill TILs per tube)
244 for the fluorescent-based analysis. This increased number of cells may account for the detection of a
245 greater number of T cell populations using the DNA-barcoded MHC multimers. In patient L013 we
246 analyzed TILs from three different tumor regions, a fragment of adjacent normal lung tissue, and
247 peripheral blood taken at the time of tumor removal. For this patient T cells responsive to the three
248 neoepitopes were most prominently detected in peripheral blood (Fig. 6b), but could be detected,
249 either significantly (ALQ), or as lower enriched populations (YSN and KVC) in T cell cultures
250 from the different regions. CMV-responsive T cell populations were detected in all regions and in
251 PBMCs. Detection of neoepitopes in peripheral blood was not feasible using combinatorial
252 fluorescently labeled MHC multimers, due to the limited samples size. Detection of such responses
253 directly from peripheral blood has the potential to significantly ease personalized T cell therapy
254 approaches and immunomonitoring.

255

256 **Assessing the functional reactivity among pMHC-responsive T cells**

257 To understand the functional capacity of pMHC responsive T cells, we combined the assessment of
258 cytokine production following target cell stimulation and the binding to pMHC multimers. In three
259 healthy donor PBMC samples we analyzed for functional reactivity towards a pool of CMV, EBV,

260 and FLU-derived peptides (CEF pool) using intracellular cytokine staining (ICS) and subsequently
261 stained with a library of 328 DNA-barcoded pMHC multimers (Supplementary Table 9 and
262 Supplementary Methods). T cells were sorted solely based on their cytokine secretion profile, INF γ
263 and TNF α secretion (ICS^{pos}) or no cytokine secretion (ICS^{neg}), but the pMHC associated DNA
264 barcode could retrospectively reveal the antigen specific composition of these different cell-subsets.
265 Functional reactivity was observed for all virus responsive T cells previously detected in these
266 PBMCs. 8 in 11 virus specific T cell populations displayed full functionality, with none of these T
267 cells detected in the ICS^{neg} fraction (Fig. 7a). Using the same principle we analyzed for functional
268 reactivity of TILs towards autologous tumor cells in a melanoma patient (Fig. 7b-c). We detected
269 eight T cell populations responsive to melanoma-associated HLA-A*0201-restricted T cell
270 epitopes. These T cell populations were either exclusively or predominantly detected in the ICS^{pos}
271 populations, indicating antigen processing and presentation on tumor cells and functional
272 capabilities among the epitope-specific TILs. In contrast, the four virus-restricted T cell populations
273 present in this TIL product were detected only in the ICS^{neg} population, as expected, since the virus
274 responsive T cells would not have met their target on autologous tumor cells. These analyses prove
275 the feasibility for combining T cell detection using large libraries of DNA-barcoded pMHC
276 multimers with assessment of functional responsiveness upon target recognition.

277

278 **DISCUSSION**

279 We report herein a technology that allows multi-parallel detection of >1000 different antigen-
280 specific CD8 T cell populations in a single sample using DNA-barcoded MHC multimers. The
281 potential complexity of DNA barcode tags exceeds $10^{8 \text{ ref10}}$, and the functional limitations rely on
282 the ability to achieve specific TCR-pMHC interactions within a very large pool of different pMHC
283 multimers. Limitations in terms of maximal number or concentration of MHC multimers hindering

284 specific TCR-pMHC multimer interactions have not been reached. The complexity may likely be
285 extended to libraries of 10,000-100,000 MHC multimers, but this will depend to some extent on the
286 automation of MHC multimer production, high-throughput peptide synthesis and microfluidics
287 systems ensuring both efficient pMHC interaction with all T cells in a given sample and that
288 specific interaction of the TCR with its cognate pMHC recognition motif will not be outmatched by
289 numerous CD8:MHC constant region interactions.

290 The detection of antigen-specific CD8 T cells using DNA-barcoded MHC multimers provides a
291 detection limit similar to that of fluorescence-based readouts and correlates tightly with the
292 frequency of antigen-responsive T cells found using combinatorial fluorescently labeled MHC
293 multimers. We could detect several T cell populations within the same sample in the frequency
294 range of 20%-0.01% of CD8 T cells (Figs. 2-4). We showed that this limit of detection may be
295 increased by increasing the number of T cells analyzed (Fig. 2e and 6a-b). Especially in the cohort
296 of tumor-derived material, we detected an increased number of pMHC-responsive T cell
297 populations when the reactivity was assessed using the DNA-barcoded MHC multimers compared
298 to the fluorescently labeled MHC multimers. This increased detection of specific T cells may reflect
299 that T cell detection based on DNA barcode-labeled MHC multimers provides less dependence on
300 stringent fluorescence-based separation, because the T cell specificities are determined by
301 sequencing the pMHC multimer associated DNA barcode and not based on the fluorescent signal as
302 a single parameter (Supplementary Fig. 5a-b). This characteristic may allow enhanced detection of
303 low-avidity T cells, since the DNA barcode label is readily recovered from those T cells that bind
304 specifically to the MHC multimer reagents even when they only obtain a marginally higher
305 fluorescent intensity than the MHC multimer negative population, but are still included in the sorted
306 fraction of T cells. Tumor-reactive T cells, especially those recognizing shared self-peptide
307 epitopes, often demonstrate lower affinity for the pMHC recognition motif compared to virus-

308 specific T cells²⁶. Additionally, the use of the dextran backbone, providing a higher-order
309 multimer, as opposed to the streptavidin-based tetramerization conventionally used for
310 combinatorial fluorescently labeled MHC multimers, may further assist the detection of low-avidity
311 T cells^{27,28}. Dextran reagents are however available carrying only three different fluorescent labels
312 and are therefore not optimal for multiplex fluorescently-based assays.

313 In numerous diseases, it is crucial to assess T cell recognition at the relevant disease site using, for
314 example, tissue biopsies. Due to the complexity of the present technology, we allow assessment of
315 T cell reactivity toward large libraries of MHC multimers even in small-size biological samples.
316 Within two melanoma patients, we compared T cell reactivity directly from tissue digest with in
317 vitro-expanded T cell product and peripheral blood before and after TIL adoptive transfer (Fig. 5a-
318 b). We prove the feasibility for detection of tumor-reactive T cells in small biological samples,
319 which allow interrogation of the dynamics in T cell recognition across large peptide libraries.
320 Previous results showed loss of antigen recognition after in vitro expansion of T cells¹³, which
321 indicates that direct ex vivo assessments of T cell reactivity will provide novel insights into T cell
322 recognition at the disease site. For diagnostic purposes and for response-to-therapy evaluations,
323 peripheral blood samples would be the preferable material to analyze due to the ease of access.
324 However, the inadequate complexity and the limit of detection of MHC multimer-based analysis
325 previously prohibited direct detection of tumor antigen-responsive T cells from peripheral blood
326 using large peptide libraries. Here, we showed the feasibility of detecting neoepitope-reactive T
327 cells when screening peripheral blood with large libraries of pMHC multimers selected based on
328 personalized tumor mutagenome analyses (Fig. 6a-b). Screening for T cell reactivity against large
329 libraries of neoepitopes using the DNA-barcoded MHC multimer one-pot approach led to enhanced
330 detection of neoepitope directed T cell reactivity compared to the use of combinatorial fluorescently
331 labeled MHC multimers that required testing of numerous parallel samples.

332 We demonstrate that it is feasible to combine the use of DNA-barcoded MHC multimers with
333 functional analysis. Upon peptide or tumor cell stimulation we selected cytokine-secreting T cells
334 and showed that functional capacity and target recognition can be assessed across large peptide
335 libraries (Fig. 7). Such functional assessments are valuable for understanding which antigens form
336 potential tumor rejection epitopes. A future strategy could combine DNA-barcoded MHC multimer
337 staining with single-cell sorting and gene expression profiling approaches²⁹⁻³¹. The analyses
338 presented herein, based on bulk assessments of T cell populations, largely reflect the information
339 that can be obtained from bulk analyses using fluorescently labeled pMHC multimers (Fig. 2-7), but
340 represents a quantitative leap in the number of potential epitopes that can be assessed in parallel.
341 This in turn offers great potential for advancing T cell assessments to genome-wide levels.
342 Moreover, it will allow such screens to be conducted not only on in vitro-expanded material but
343 also directly on tissue biopsies and peripheral blood.

344

345 **Author Contributions**

346 AKB conceived the concept, designed and performed experiments, analyzed data, made figures and
347 wrote the manuscript; AMM designed the in silicon analysis, analyzed data, and made figures; RL,
348 SKS, and SRA, produced MHC monomers, designed and performed experiments, analyzed data and
349 revised the manuscript; LS performed experiments, MD and IMS, provided patients material and
350 generated tumor cell lines, discussed data; PtS provided administrative support, flow facility and
351 production of MHC monomers; AF, SAQ, provided patients material and peptides from NSCLC;
352 NMG, RR, CAS, identified tumor mutagenome and predicted NSCLC-associated neoepitopes; ZS
353 and ACE, designed the in silicon analysis, and guided data analyses; SNJ designed DNA barcodes,
354 conceived the concept, guided data analyses and revised the manuscript; SRH conceived the
355 concept, designed experiments, analyzed data and wrote the manuscript.

356

357 **Acknowledgments**

358 We would like to thank Ulla Kring Hansen, Anna Burkal and Tina Seremet for technical assistance,
359 and Ton Schumacher, Netherlands Cancer Institute for scientific discussions and sharing of MHC
360 expression plasmids. The work was funded by The Danish Cancer Society, The Lundbeck
361 Foundation and The Danish Research Council.

362 **Competing financial interests:** The technology is patented by the authors (SRH, AKB, and SNJ),
363 the Capital Region of Copenhagen, University Hospital Herlev and licensed to Immudex.

364 **References**

- 365 1. Altman, J. D. *et al.* Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**, 94–
366 96 (1996).
- 367 2. Chattopadhyay, P. K. & Roederer, M. Cytometry: today's technology and tomorrow's
368 horizons. *Methods* **57**, 251–8 (2012).
- 369 3. Bendall, S. C. *et al.* Single-cell mass cytometry of differential immune and drug responses
370 across a human hematopoietic continuum. *Science* **332**, 687–96 (2011).
- 371 4. Hadrup, S. R. *et al.* Parallel detection of antigen-specific T-cell responses by
372 multidimensional encoding of MHC multimers. *Nat. Methods* **6**, 520–526 (2009).
- 373 5. Newell, E. W., Klein, L. O., Yu, W. & Davis, M. M. Simultaneous detection of many T-cell
374 specificities using combinatorial tetramer staining. *Nat. Methods*. **6**, 497–499 (2009).
- 375 6. Newell, E. W. *et al.* Combinatorial tetramer staining and mass cytometry analysis facilitate
376 T-cell epitope mapping and characterization. *Nat. Biotechnol.* **31**, 623–9 (2013).
- 377 7. Newell, E. W., Sigal, N., Bendall, S. C., Nolan, G. P. & Davis, M. M. Cytometry by time-of-

- 378 flight shows combinatorial cytokine expression and virus-specific cell niches within a
379 continuum of CD8⁺ T cell phenotypes. *Immunity* **36**, 142–52 (2012).
- 380 8. Robins, H. S. *et al.* Comprehensive assessment of T-cell receptor beta-chain diversity in
381 alphabeta T cells. *Blood* **114**, 4099–107 (2009).
- 382 9. Davis, M. M. & Bjorkman, P. J. T-cell antigen receptor genes and T-cell recognition. *Nature*
383 **334**, 395–402 (1988).
- 384 10. Xu, Q., Schlabach, M. R., Hannon, G. J. & Elledge, S. J. Design of 240,000 orthogonal
385 25mer DNA barcode probes. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 2289–94 (2009).
- 386 11. Kivioja, T. *et al.* Counting absolute numbers of molecules using unique molecular identifiers.
387 *Nat. Methods* **9**, 72–74 (2011).
- 388 12. Andersen, R. S. *et al.* Parallel detection of antigen-specific T cell responses by combinatorial
389 encoding of MHC multimers. *Nat. Protoc.* **7**, 891–902 (2012).
- 390 13. Andersen, R. S. *et al.* Dissection of T-cell antigen specificity in human melanoma. *Cancer*
391 *Res.* **72**, 1642–50 (2012).
- 392 14. Lyngaa, R. *et al.* T-cell responses to oncogenic Merkel cell polyomavirus proteins
393 distinguish patients with Merkel cell carcinoma from healthy donors. *Clin. Cancer Res.* **20**,
394 1768–1778 (2014).
- 395 15. Frøsig, T. M. *et al.* Broadening the repertoire of melanoma-associated T-cell epitopes.
396 *Cancer Immunol. Immunother.* **64**, 609–20 (2015).
- 397 16. van Buuren, M. M. *et al.* HLA Micropolymorphisms Strongly Affect Peptide-MHC
398 Multimer-Based Monitoring of Antigen-Specific CD8⁺ T Cell Responses. *J. Immunol.* **192**,
399 641–8 (2014).

- 400 17. Frøsig, T. M. *et al.* Design and validation of conditional ligands for HLA-B*08:01, HLA-
401 B*15:01, HLA-B*35:01, and HLA-B*44:05. *Cytometry. A* **87**, 967–75 (2015).
- 402 18. Valmori, D. *et al.* Enhanced generation of specific tumor-reactive CTL in vitro by selected
403 Melan-A/MART-1 immunodominant peptide analogues. *J. Immunol.* **160**, 1750–8 (1998).
- 404 19. Derré, L. *et al.* A novel population of human melanoma-specific CD8 T cells recognizes
405 Melan-AMART-1 immunodominant nonapeptide but not the corresponding decapeptide. *J.*
406 *Immunol.* **179**, 7635–45 (2007).
- 407 20. Wieckowski, S. *et al.* Fine structural variations of alphabetaTCRs selected by vaccination
408 with natural versus altered self-antigen in melanoma patients. *J. Immunol.* **183**, 5397–406
409 (2009).
- 410 21. Speiser, D. E. *et al.* Unmodified self antigen triggers human CD8 T cells with stronger tumor
411 reactivity than altered antigen. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 3849–54 (2008).
- 412 22. Andersen, R. Long-lasting complete responses in patients with metastatic melanoma after
413 adoptive cell therapy with tumor-infiltrating lymphocytes and an attenuated IL-2 regimen.
414 *Clin. Cancer Res. Accept. Publ.* (2016).
- 415 23. Snyder, A. *et al.* Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N.*
416 *Engl. J. Med.* **371**, 2189–99 (2014).
- 417 24. Rizvi, N. A. *et al.* Cancer immunology. Mutational landscape determines sensitivity to PD-1
418 blockade in non-small cell lung cancer. *Science* **348**, 124–8 (2015).
- 419 25. McGranahan, N. *et al.* Clonal neoantigens elicit T cell immunoreactivity and sensitivity to
420 immune checkpoint blockade. *Science* **351**, 1463–9 (2016).
- 421 26. Aleksic, M. *et al.* Different affinity windows for virus and cancer-specific T-cell receptors:

- 422 Implications for therapeutic strategies. *Eur. J. Immunol.* **42**, 3174–3179 (2012).
- 423 27. Dolton, G. *et al.* Comparison of peptide-major histocompatibility complex tetramers and
424 dextramers for the identification of antigen-specific T cells. *Clin. Exp. Immunol.* **177**, 47–63
425 (2014).
- 426 28. Massilamany, C. *et al.* Direct staining with major histocompatibility complex class II
427 dextramers permits detection of antigen-specific, autoreactive CD4 T cells in situ. *PLoS One*
428 **9**, e87519 (2014).
- 429 29. Han, A., Glanville, J., Hansmann, L. & Davis, M. M. Linking T-cell receptor sequence to
430 functional phenotype at the single-cell level. *Nat. Biotechnol.* **32**, 684–692 (2014).
- 431 30. Dössinger, G. *et al.* MHC multimer-guided and cell culture-independent isolation of
432 functional T cell receptors from single cells facilitates TCR identification for
433 immunotherapy. *PLoS One* **8**, e61384 (2013).
- 434 31. Klein, A. M. *et al.* Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic
435 Stem Cells. *Cell* **161**, 1187–1201 (2015).

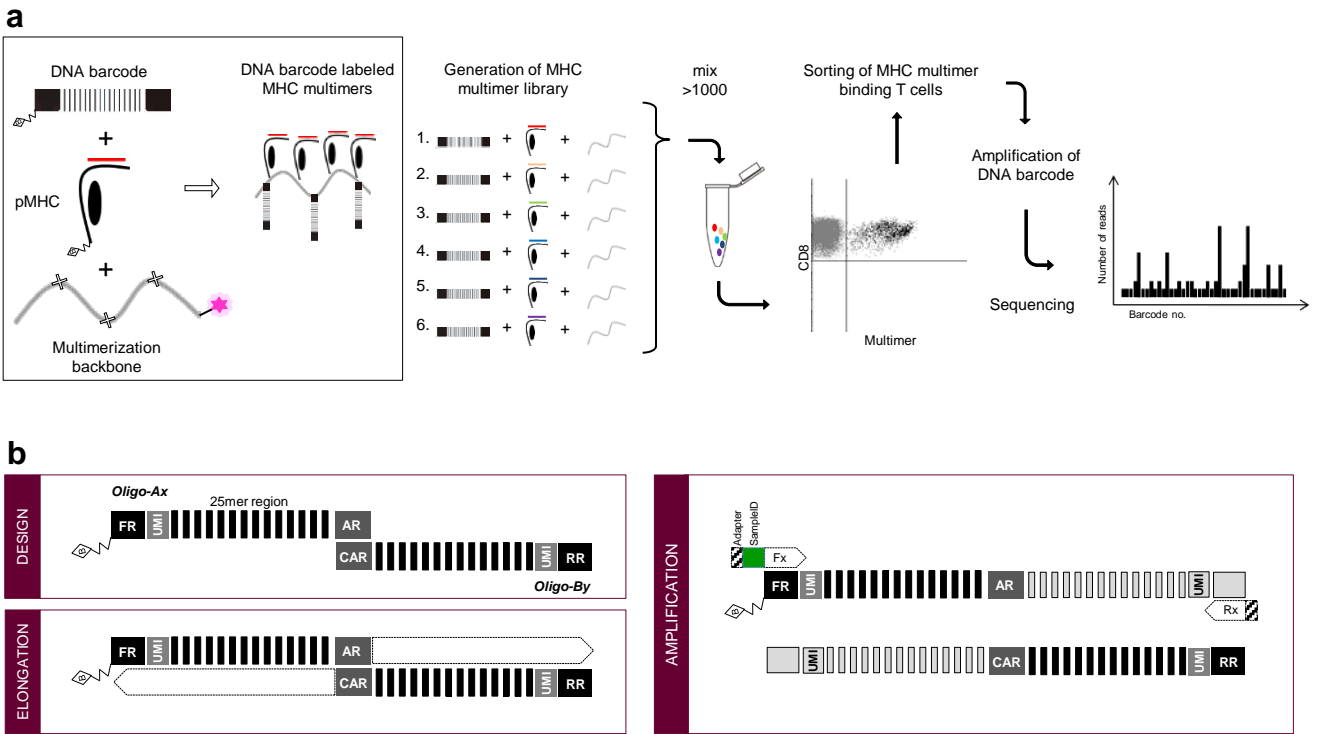


Figure 1. Preparation and use of DNA barcode-labeled MHC multimers

(a) Schematic overview showing the strategy for using DNA barcode-labeled MHC multimers for detection of antigen-specific T cells in complex cellular suspensions. Biotinylated DNA barcodes and pMHC molecules are co-attached to a PE-labeled dextran backbone carrying streptavidin; B=biotin. Each MHC multimer is assembled with a given DNA barcode, forming a tag for the corresponding specificity (1 to 1000). MHC multimer-binding T cells are isolated and sorted based on the PE label. DNA barcodes are amplified and sequenced, and the relative numbers of DNA barcode reads are used to determine the composition of antigen-responsive T cells in the sample. (b) Schematic overview of the DNA barcode design. B=biotin, FR= forward region, UMI=Unique Molecular Identifier, coding region= 25mer barcode sequence assigning pMHC specificity, AR=annealing region, CAR= complementary annealing region, and RR= reverse region. The biotinylated oligo Ax comprise a 15-nucleotide region partially complementary to oligo By. Oligos Ax and By both contain an individual 25mer oligonucleotide sequence (determined by the 'x or y') and six randomly incorporated nucleotides, providing a UMI for each synthesized oligo. Oligo A contain a forward primer region (FR), and Oligo B contain a reverse primer region (RR) (the oligonucleotide sequences are listed in Supplementary Table 1-2). Following annealing of Oligo Ax and Oligo By and prior to the attachment to the multimerization backbone, the oligos are elongated to obtain their double-stranded form. After the isolation of MHC multimer-binding T cells, the DNA barcodes are amplified by PCR (Supplementary Table 3). The forward primer is flanked by a 5' sample identifier sequence (sampleID), and both the forward and reverse primers encodes a 5' sequencing adaptor sequence (IonTorrent, A-Key and P1-key, respectively) (Supplementary Table 3).

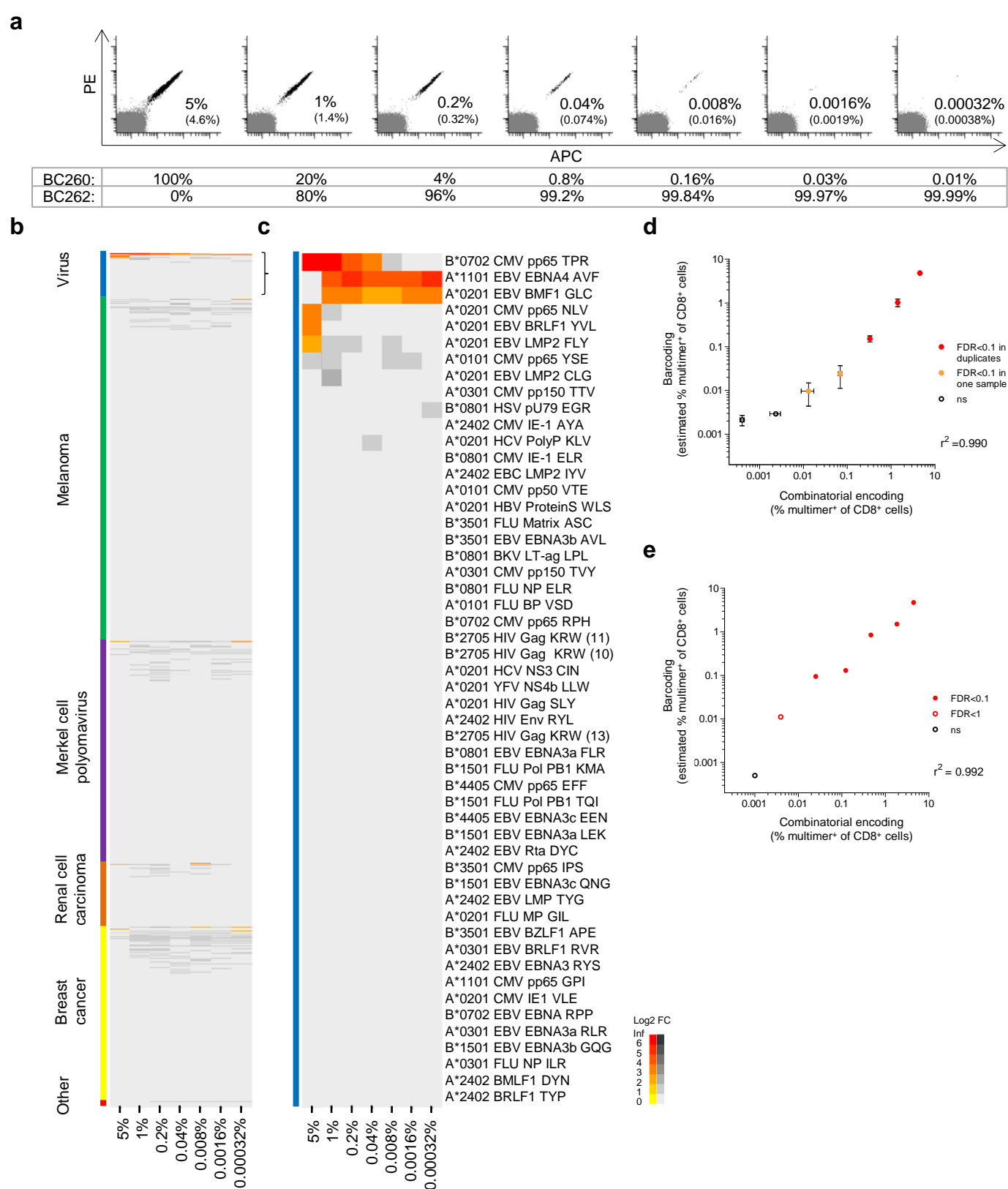


Figure 2. Dynamic range and limit of detection of DNA-barcoded MHC multimers

(a) Fluorescent multimer-based assessment of seven samples with various proportions of B*0702 CMV_{TPR}-specific T cells, theoretically: 5%, 1%, 0.2%, 0.04%, 0.008%, 0.0016% and 0.00032% of CD8 T cells. Samples are generated from BC260 PBMCs mixed with HLA-B7-negative BC262 at fivefold dilutions. Total percentage of cells derived from each donor is indicated below the corresponding dot plot. The flow-based percentages of MHC multimer⁺ CD8 T cells are given in brackets (detection limit: ≥ 10 events and $> 0.002\%$ of CD8 T cells). (b) Heatmap representing the DNA barcode-based analysis of samples from (a). Each sample was screened with a panel of 1031 pMHC multimers. The heatmap shows changes in read proportions compared to background levels (Log₂FC, see Supplementary Methods, Statistical analysis). Each column represents the reads associated with a given sample and each row a given antigen specificity, i.e. reads mapped to the DNA barcode associated with the corresponding pMHC. Epitopes are grouped based on their antigen origin. Within each antigen group rows are sorted based on Log₂FC, highest to lowest, compared to baseline samples. Yellow-red or gray scaling, respectively, indicates statistically significant (FDR < 0.1%) or insignificant (FDR $\geq 0.1\%$) number of reads. A duplicate analysis is shown in Supplementary Figure 2. (c) Magnification focused on the virus-derived peptides. First row represents the target specificity B*0702 CMV_{TPR} present in BC260. 2nd and 3rd rows below are T cell responses from the HLA-B*0702 negative donor BC262, followed by three lower-frequent responses that are present in BC260. (d, e) Correlations between the frequency of HLA-B*0702 CMV_{TPR}-specific T cells determined by analyzing the same samples using combinatorial fluorescently labeled pMHC multimers or a panel of 1031 DNA-barcoded pMHC multimers, (d) when staining 2×10^6 PBMCs per sample or (e) 10×10^6 PBMCs per sample. Error bars represent range of duplicates. All pMHC multimers are 'dextramers'. A similar experiment using another pMHC multimer library is shown in Supplementary Figure 3.

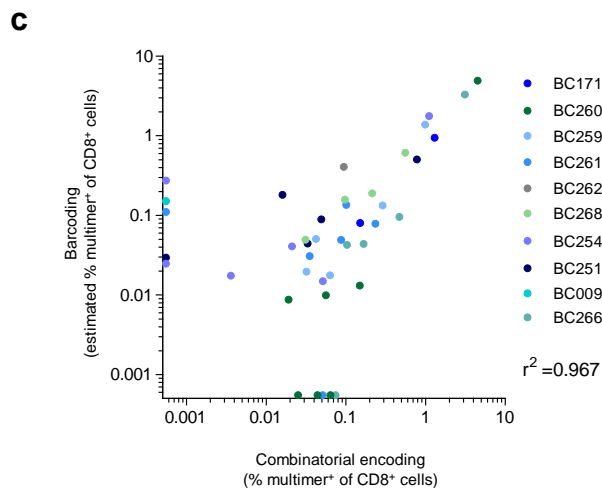
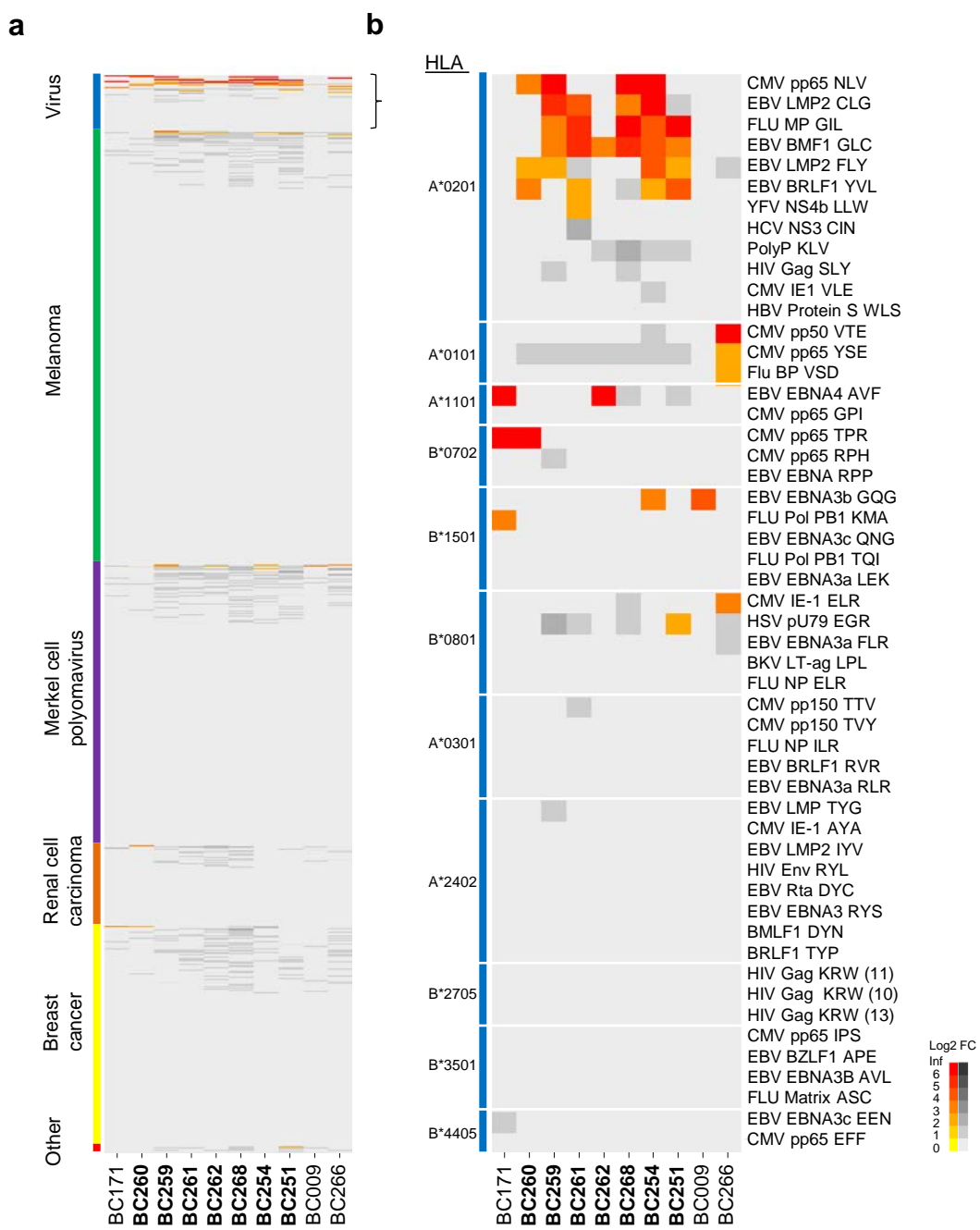
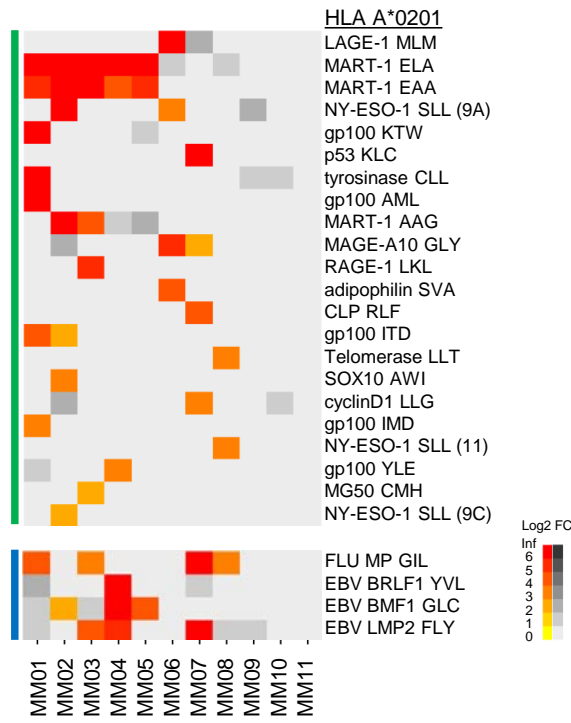
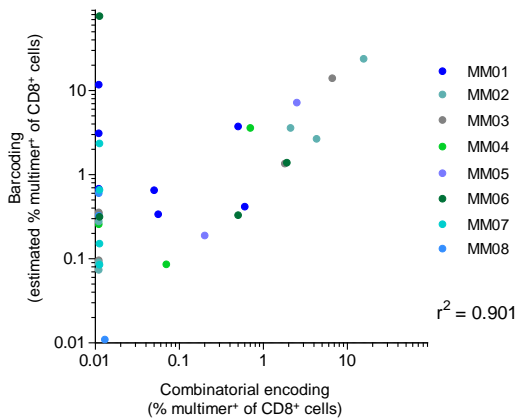


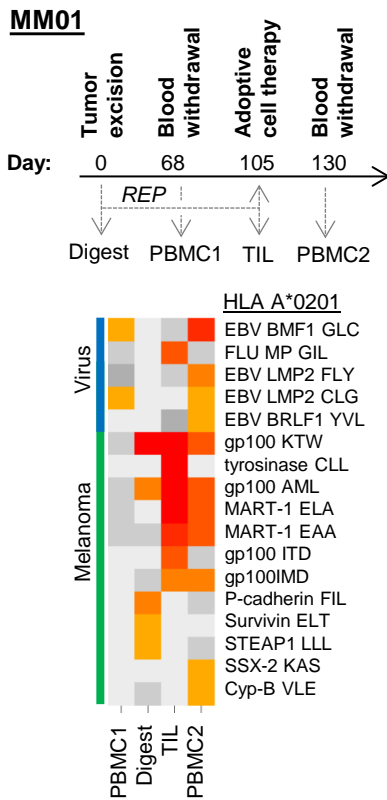
Figure 3. High-throughput assessment of T cell reactivity using large peptide libraries

(a) DNA barcode-based analysis of 10 different healthy donor PBMC samples ($1-2 \times 10^6$ PBMCs) using 1031 pMHC multimers, each carrying individual barcodes. The heatmap is organized as in Figure 2b, each column represents one donor. Donors marked in bold are all HLA-A*0201. Further details on HLA-types of all donors are presented in Supplementary Table 7. (b) Magnification focused on the virus-derived peptides (52 epitopes). Rows representing antigen specificities are grouped according to HLA-type and sorted within each group based on Log2FC. Each sample shown here was analyzed once, but replicates with six of the same donors using another pMHC library, shown in Supplementary Figure 4. (c) Correlations between antigen-specific T cell frequencies, from analyses of T cell responses in 10 healthy donors using either combinatorial fluorescently labeled pMHC multimers (x-axes) or 1031 DNA-barcoded pMHC multimers (y-axes). Each dot represents one specificity. Only T cell populations that fulfilled the significance criteria for DNA barcode assessment ($FDR < 0.1\%$) or the threshold for fluorescent-based analysis (≥ 10 events and $> 0.002\%$ of CD8 T cells) are plotted. All specificities included in the plot were tested using both a combinatorial encoding analysis and DNA-barcoded MHC multimers. Dots plotted on the axes are nonsignificant for one of the methods.

a**b****c****Figure 4. High-throughput assessment of tumor-reactive T cells**

(a) Heatmap representing the barcode-based analysis of 11 different samples of in vitro-expanded tumor-infiltrating lymphocytes (TILs) using a library of 167 HLA-A*0201 melanoma-associated pMHC multimers and 8 virus-derived pMHC multimers (175 pMHC library, Supplementary Table 8). The heatmap is organized as in Figure 2b. Donor MM9, MM10 and MM11 carries the HLA-A*0205 subtype (Supplementary Table 7). Each TIL culture were analyzed once. Three TIL cultures were reanalyzed with a larger pMHC library, with similar results (data not shown) (b) Magnification focusing on significant responses among both categories of peptides. (c) Correlations between antigen-specific T cell frequencies determined across the 11 different TIL samples from melanoma patients analyzed using combinatorial fluorescently labeled pMHC multimers (x-axes) or DNA-barcoded pMHC multimers (y-axes). Each dot represents one specificity. Only T cell populations that fulfilled the significance criteria described in Figure 3c were plotted. All specificities were tested using both a combinatorial fluorescently labeled pMHC multimers and DNA-barcoded pMHC multimers. 0.5-2x10⁶ TILs were analyzed per sample in both methods.

a



b

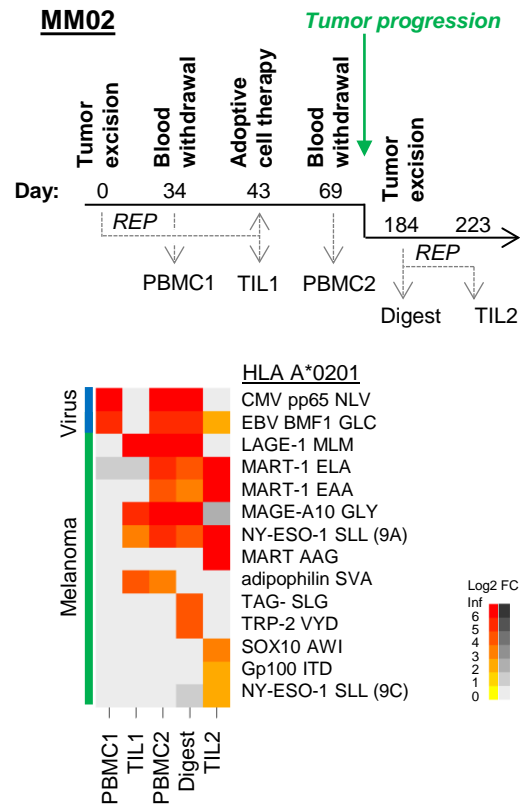


Figure 5. T cell assessment of limited biological samples

(a, b) Analysis of dynamic changes in T cell response to melanoma-associated antigens (175 pMHC library, Supplementary Table 8) before and after TIL adoptive cell transfer in two patients with metastatic melanoma. A timeline of sample collection and TIL adoptive transfer are presented for each patient together with a heatmap focusing on T cell specificities detected in any of the samples from (a) patient MM01 and (b) patient MM02. The heatmaps are organized as described in Figure 2b. All samples were analyzed once.

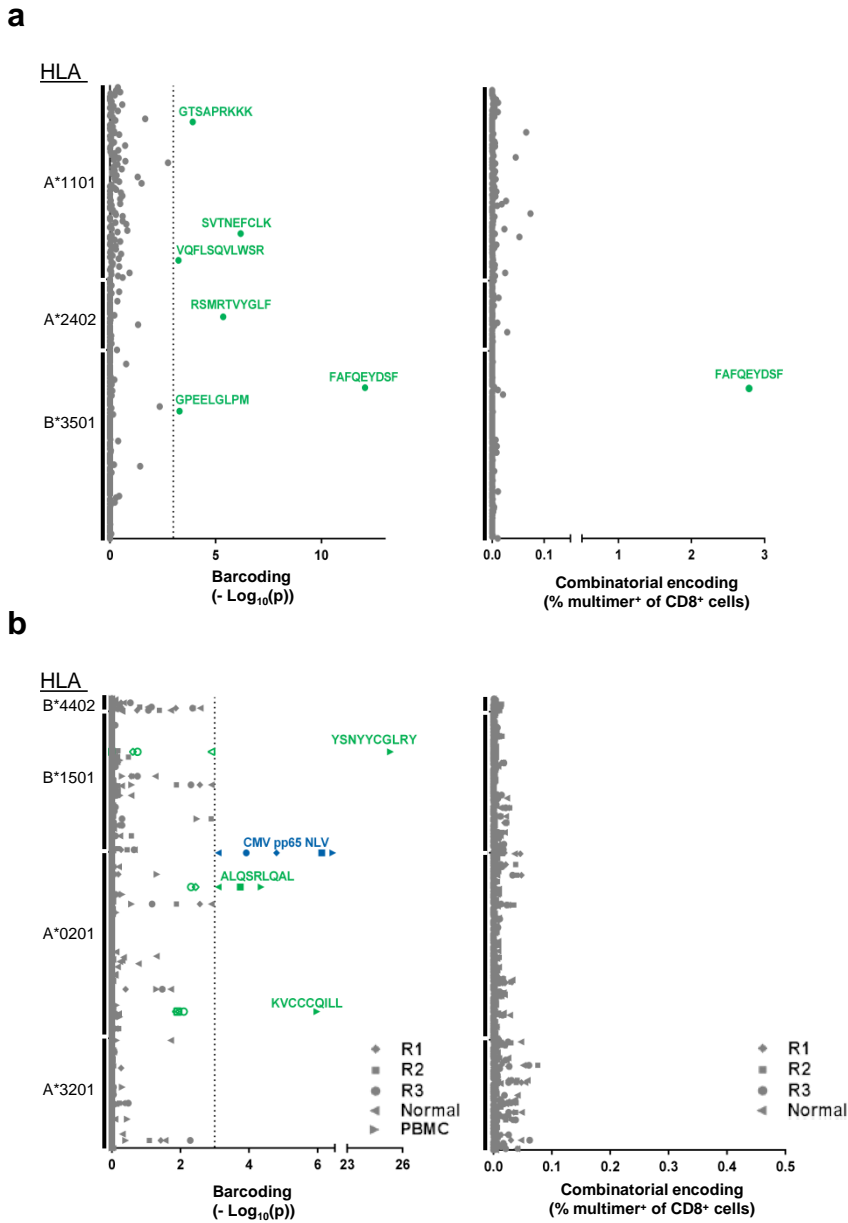
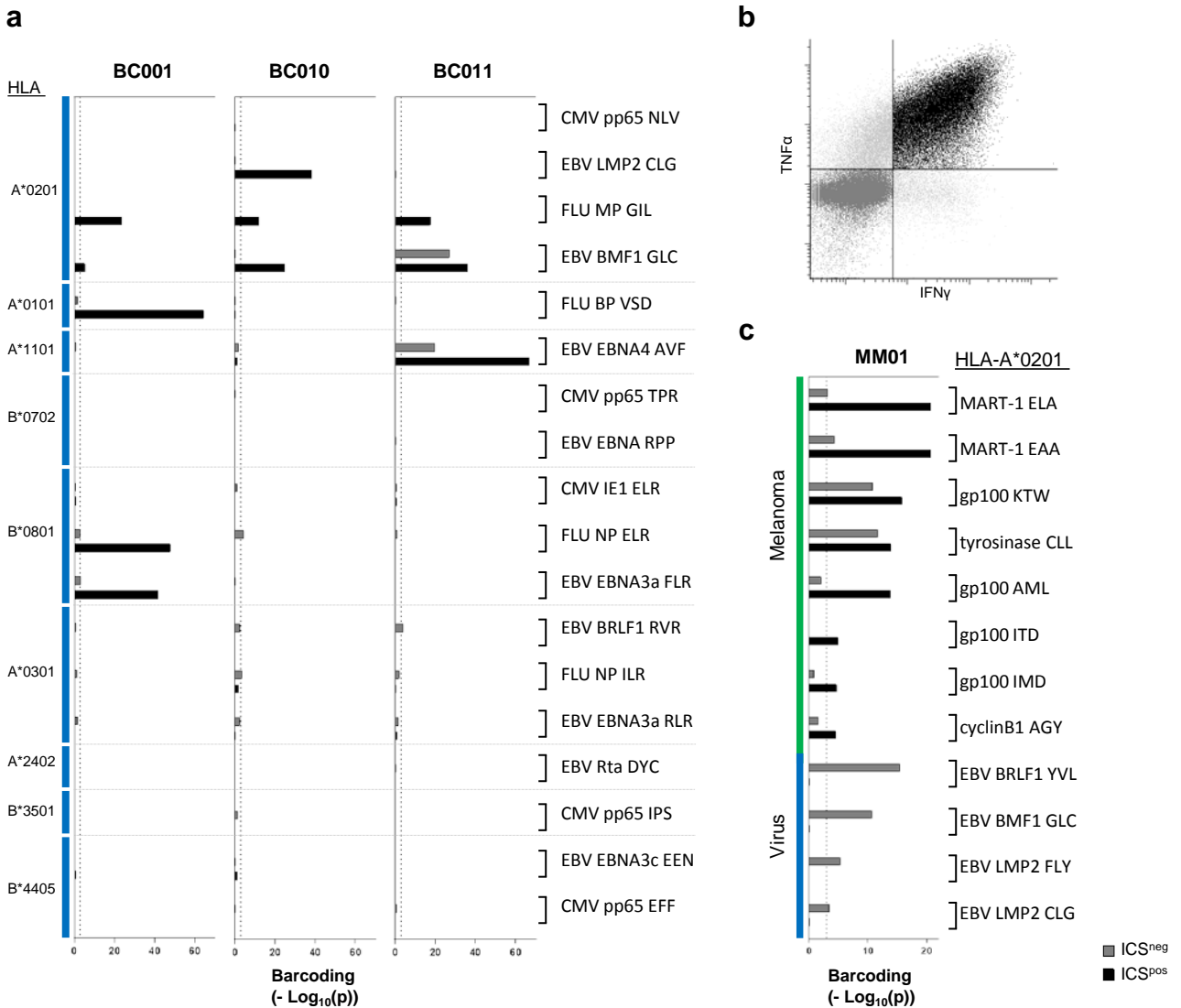


Figure 6. Detection of neopeptide responsive T cells in lung cancer

(a) Screening for T cell recognition of 288 predicted neopeptides and 10 HLA-matched virus-derived peptides in T cells expanded from a resected lesion in patient L011 using either combinatorial fluorescently labeled MHC multimers (1×10^6 viable cells per tube in 9 tubes) or DNA-barcoded MHC multimers (5×10^6 viable cells per tube in 1 tube). Results from all 288 pMHC multimers included in the screening are plotted (x-axis). Data plotted on the y-axis is the percentage of MHC multimer positive T cells of total CD8 T cell for combinatorial fluorescently labeled MHC multimers, or $-\log_{10}(p)$ (in respect to the pMHC associated DNA barcode) for the DNA-barcoded MHC multimer analyses (average of duplicates). Dotted line at $y=3$ ($-\log_{10}(0.001)$) represent the selected threshold of $FDR < 0.1\%$. The FAFQEYDSF specific response was confirmed by multiple ($n>3$) additional fluorescent-based analyses²¹. (b) Screening for T cell recognition of 417 predicted neopeptides and six HLA-matched virus derived epitopes in samples of different origin from patient L013 using either combinatorial fluorescently labeled MHC multimers ($1-2 \times 10^6$ viable cells per tube in 13 tubes) or DNA-barcoded MHC multimers ($4-8 \times 10^6$ viable cells per tube in 1 tube). Samples were derived from in vitro expanded T cells from three resected tumor regions and from one normal lung region. PBMCs were only analyzed with DNA-barcoded MHC multimers (2×10^6 viable cells per tube). Results from all 423 pMHC multimers included in the screenings are plotted (x-axis). Y-axis data is plotted as in (a). Each L013-derived sample were analyzed once. Open symbols represents an epitope recognized in at least one of the remaining four samples with $FDR < 0.1$.



1 **EXPERIMENTAL PROCEDURES**

2

3 **Cell samples**

4 Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated from whole blood
5 by density centrifugation on Lymphoprep (Axis-Shield PoC), and cryopreserved at -150 °C in fetal
6 calf serum (FCS; Gibco) + 10% DMSO.

7 Material from metastatic melanoma patients: TILs were obtained from resected tumor lesions from
8 individuals with AJCC stage IV melanoma. Tumor lesions were resected following surgical
9 removal. Tumor fragments from one metastatic lesion (1–3 mm³) were either enzymatically
10 digested overnight and immediately cryopreserved (uncultured TILs, see below), or cultured
11 individually in complete medium (RPMI with 10% human serum, 100 U/ml penicillin, 100 µg/ml
12 streptomycin, 1.25 µg/mL fungizone (Bristol-Myers Squibb) and 6000 U/ml IL-2) at 37 °C and 5%
13 CO₂, allowing TILs to migrate into the medium. TILs were expanded to reach >50x10⁶ total cells
14 originated from approximately 48 individual fragments, which had expanded to confluent growth in
15 2-mL wells and eliminated adherent tumor cells (average of approximately 2x10⁶ cells per well
16 from each tumor fragment). TIL cultures were further expanded using a standard rapid expansion
17 protocol (REP) as previously described³². Briefly, TILs were stimulated with 30 ng/mL anti-CD3
18 antibody (OKT-3; Ortho Biotech) and 6000 U/mL IL-2 in the presence of irradiated (40 Gy)
19 allogenic feeder cells (healthy donor PBMCs) at a feeder:TIL ratio of 200:1. Initially, TILs were
20 rapidly expanded in a 1:1 mix of complete medium and REP medium (AIM-V [Invitrogen] + 10%
21 human serum, 1.25 µg/mL fungizone and 6000 U/mL IL-2), but after seven days complete medium
22 and serum were removed stepwise from the culture by adding REP medium without serum to
23 maintain cell densities around 1x10⁶–2x10⁶ cells/mL. TIL cultures and tumor cell lines were
24 cryopreserved at -150 °C in human serum + 10% DMSO.

25 Uncultured TILs were established as described previously³³. Briefly, tumor fragments freshly

26 dissected from the same metastatic lesion as the expanded TILs were resuspended in RPMI media
27 containing 1 mg/mL of collagenase type IV (Sigma-Aldrich) and 0.0125 mg/mL dornase alpha
28 (Pulmozyme, Roche). After overnight digestion at room temperature under gentle sample agitation,
29 single cell suspensions were cryopreserved at -150 °C in human serum + 10% DMSO.
30 Blood samples from patients with melanoma were collected at different time points, i.e. before and
31 after infusion of rapidly expanded TILs in a phase II trial²². Clinical outcome of patient MM01 and
32 patient MM02 is reported elsewhere (the two patients are identified as M37 and M43,
33 respectively)²². Tissue typing was conducted by HLA amplification and sequencing (IMGM
34 Laboratories GmbH) or by PCR and flow cytometry (results listed in Supplementary Table 7). All
35 procedures were approved by the Scientific Ethics Committee for the Capital Region of Denmark.
36 Written informed consent was obtained according to the Declaration of Helsinki.

37 Material from NSCLC patients:

38 Samples for sequencing were obtained from patients (L011 and L013) diagnosed with non-small
39 cell lung cancer (NSCLC) who underwent definitive surgical resection prior to receiving any form
40 of adjuvant therapy, such as chemotherapy or radiotherapy. Informed consent allowing for genome
41 sequencing had been obtained. All samples were collected from University College London
42 Hospital, London (UCLHRTB 10/H1306/42). L011 were classified with CK7+/TTF1+
43 adenocarcinoma (LUAD), L013 with squamous cell carcinoma (LUSC). Patient material was
44 processed as described in detail in McGranahan et al, 2016²⁵.

45 Tumor Processing: Up to five regions from a single tumor mass, separated by 1 cm intervals, and
46 adjacent normal tissue were resected, and snap-frozen. Peripheral blood was collected at the time of
47 surgery. Approximately 5x5x5 mm snap-frozen tumor tissue and 500 µL blood were used for
48 genomic DNA extraction. Multi-region Whole-Exome Sequencing and variant calling for each
49 tumor region and matched germ-line from patient L011 and L013 was conducted as described

50 previously^{34,35}. Patients were serotyped and simultaneously genotyped using Optitype³⁶, which
51 produced concordant results. Patients the following HLA-A and B genotype: L011; HLA-A*2402,
52 A*1101, B*3501 and B*4901, and L013; HLA-A*0201, A*3201, B*4402 and B*1501. For our T
53 cell analyses we were able to cover 7 out of the 8 HLA A and B molecules (HLA-B*4901 was not
54 included).

55 Isolation and in-vitro expansion of tumor-infiltrating lymphocytes (TILs) for L011 and L013:

56 Directly after tumor resection the sample was divided into regions. Samples were digested
57 enzymatically, mechanically dissociated and TILs were expanded using a REP protocol, all are
58 described in detail in McGranahan et al, 2016²⁵.

59

60 **Generation of DNA barcodes and dextran conjugation**

61 Oligonucleotides containing distinct 25mer nucleotide sequences (from Xu et al. 2009) were
62 purchased from DNA Technology (Denmark). Oligonucleotides modified with a 5' biotin tag (oligo
63 A) were joined to unmodified, partially complementary oligonucleotides (oligo B) to generate
64 >1000 unique double-stranded AxBy DNA barcodes (sequences are listed in Supplementary Table
65 1 and 2). Combinations of A and B oligos (one of each) were mixed with 5x Sequenase Reaction
66 Buffer mix (PN 70702, Affymetrix) to final concentrations of 26 μ M (Oligo A) and 52 μ M (Oligo
67 B), respectively; heated to 65 °C for 2 min; and allowed to anneal by cooling slowly to <35 °C over
68 15-30 min. The annealed oligo A's and B's were elongated to create double-stranded AxBy DNA
69 barcodes by adding Sequenase polymerase (70775Y, Affymetrix), 20 μ M DTT and 800 μ M or 72
70 μ M dNTPs, followed by incubation for 5-10 min at RT. Elongated AxBy barcodes were diluted in
71 nuclease-free water + 0.1% Tween to 2.17 μ M (with respect to the A oligo) and stored at -20 °C.
72 5' biotinylated AxBy DNA barcodes were attached to PE- and streptavidin-conjugated dextran
73 (from Immudex). The amount of DNA barcode was titrated for each lot of dextran conjugate to

74 ensure that the MFI was unaffected or affected only minimally when staining antigen-specific T
75 cells with DNA-barcoded multimers compared to non-barcoded multimers generated from the same
76 dextran-conjugate lot. For the dextran-conjugates applied during this study (6-7 SA per dextran),
77 the amount of attached DNA barcode was 4-6 times less than the amount that would lead to a
78 complete loss of antigen-specific interactions between barcoded multimers and T cells, i.e., an
79 estimated two DNA barcodes per dextran backbone. DNA barcodes were attached by mixing with
80 dextran-conjugate, followed by incubation, 30 min at 4 °C. DNA barcode-assembled dextran-
81 conjugates were stored for up to one week at 4 °C.

82

83 **Peptides and MHC monomer production**

84 Peptides were purchased from Pepscan (Pepscan Presto) and dissolved to 10 mM in DMSO. UV-
85 sensitive ligands were synthesized as previously described³⁷⁻³⁹. Recombinant HLA-A0101, HLA-
86 A0201, HLA-A0301, HLA-A1101, HLA-A2402, HLA-A3201, HLA-B0702, HLA-B0801, HLA-
87 B1501, HLA-B2705, HLA-B3501, HLA-B4402 and HLA-B4405 heavy chains and human β 2
88 microglobulin light chain were produced in Escherichia coli. HLA heavy and light chains were
89 refolded with UV-sensitive ligands and purified as described in Hadrup et al., 2009⁴¹. Specific
90 peptide-MHC complexes were generated by UV mediated peptide MHC exchange^{17,38,39,42}. Nine of
91 1031 pMHC monomers were refolded immediately with antigenic peptide as described in Garboczi,
92 Hung, & Wiley, 1992⁴³.

93 Identification of Putative Neoantigens related to NSCLC patients, L011 and L013:

94 Identified non-silent mutations were used to generate a list of 9-11 amino acid long peptides with
95 the mutated amino acid represented in each possible position. The binding affinity of every mutant
96 peptide and its corresponding wild-type peptide to the patient's germline HLA alleles was predicted

97 using netMHCpan-2.8^{44,45}. Candidate neo-antigens were identified as those with a predicted binding
98 strength of <500 nM. We predicted 288 potential HLA class I binding peptides for L011, with the
99 relative distribution of 45 HLA-A*2402, 121 A*1101 and 122 B*3501 binding peptides. For L013
100 we predicted a total of 417 potential HLA class I binding neoepitopes, including 173 HLA-A*0201,
101 104 A*3201, 10 B*4402 and 130 B*1501 binding peptides. All predicted neoepitopes were
102 synthesized. Peptide binding was not validated experimentally, as the described technology allow
103 screening of the full library without further selection. Previous experimental validation of peptide-
104 HLA binding has shown that approximately 50% of the predicted peptides will bind to HLA
105 molecules with medium to high affinity (defined as >60% of a comparable virus-derived T cell
106 epitope)^{14,15}

107 Other peptide libraries included:

108 In the 1031 peptides library we included peptide libraries associated to Merkel Cell Carcinoma and
109 Breast cancer. Both of these libraries were selected as previously described¹⁴; using a combination
110 of SYFPEITHI⁴⁶ and netMHC^{44,45}. Thresholds applied: > 19 in SYFPEITHI and < 1000 in
111 netMHC. All predicted peptides were experimentally validated for MHC binding as described in
112 Rodenko et al.³⁸. Peptide libraries associated to Melanoma and RCC included previous published T
113 cell epitopes, identified as described in¹³.

114

115 **Generation of DNA barcode-labeled peptide-MHC multimer libraries**

116 Unoccupied SA-binding sites on the DNA barcode-assembled dextran conjugates were used for the
117 co-attachment of biotinylated pMHC molecules. 2.3 nmol pMHC monomer was mixed with
118 160×10^{-15} mol DNA-barcoded dextran-conjugate and incubated 30 min at RT. MHC multimers
119 were diluted in PBS with 5.2 μ M D-biotin (Avidity, Bio200) to 50 μ g/mL and incubated 20 min on
120 ice. DNA-barcoded MHC multimers were either stored for up to 5 days at 4 °C (PBS supplemented

121 with NaN₃, final concentration 0.02% (w/v)) or for up to two months at -80 °C (PBS supplemented
122 with glycerol and BSA, final concentrations 5% and 0.5%, respectively).
123 Immediately before staining barcode-labeled MHC multimers were centrifuged, 5 min at 3300 g,
124 and pooled (2.3 nmol of each pMHC/sample) to enable the detection of multiple T cell responses in
125 parallel. If necessary, the volume of the reagent pool was reduced up to 50x by ultrafiltration to
126 obtain a final volume of approx. 80 µL of MHC multimers: centrifugal concentrators (Vivaspin 20,
127 100,000 Dalton, Sartorius) were saturated in PBS + 5 % BSA by centrifugation until >10 ml had
128 passed through the membrane. Concentrators were washed twice, each time by centrifuging 20 ml
129 PBS through the membrane. The pooled multimers were loaded onto the concentrator and
130 centrifuged, 3300 g, 4 °C, to reach a final concentration of 23 µM for each pMHC in the staining
131 reaction. Any aggregates in the MHC multimer reagent pool were sedimented by centrifugation, 5
132 min 3300 g before addition to the cell sample. An aliquot of approx. 5 µl of the MHC multimer
133 reagent pool was stored at -20 °C for baseline analysis.

134

135 **Generation of fluorescently labeled pMHC tetramers**

136 MHC tetramers were assembled as previously described^{4,12} with nine fluorescent SA conjugates
137 (SA-PE, SA-APC, SA-PE-Cy7, SA-BV421, SA-BV510, SA-BV605, SA-BV650, SA-PE-CF594,
138 SA-BUV395) (BioLegend, Nordic Biosite, Denmark) to allow two-dimensional staining with
139 fluorescent labels of up to 36 T cell receptor specificities in parallel.

140

141 **Peptide-MHC multimer staining**

142 Cryopreserved PBMCs and TILs were thawed and washed in RPMI + 10% FCS. Cells were washed
143 in a barcode-cytometry buffer (PBS + 0.5% BSA + 100ug/ml herring DNA + 2mM EDTA) and

144 incubated 30 min, 37 °C in the presence of 50 nM dasatinib. 2×10^5 - 3×10^6 cells (except when
145 specifically stated) were incubated, 15 min, 37 °C, with pooled DNA-barcoded multimers in a total
146 volume of 100 μ l (final concentration of each distinct pMHC, 23 μ M). Next a 5x antibody mix
147 composed of CD8-PerCP (Invitrogen MHCD0831) (final dilution 1/50), dump channel antibodies:
148 CD4-FITC (BD 345768) (final dilution 1/80), CD14-FITC (BD 345784) (final dilution 1/32),
149 CD19-FITC (BD 345776) (final dilution 1/16), CD40-FITC (Serotech MCA1590F) (final dilution
150 1/40), CD16-FITC (BD 335035) (final dilution 1/64) and a dead cell marker (LIVE/DEAD Fixable
151 Near-IR; Invitrogen L10119) (final dilution 1/1000) was added and incubated 30 min, 4 °C. Cells
152 were washed twice in barcode-cytometry buffer and if not acquired immediately, fixed in 1%
153 paraformaldehyde. If the cells were not acquired within 24 h they were washed twice more and
154 resuspended in barcode-cytometry buffer. Cells were acquired within a week after multimer
155 staining.

156 Generation of spiked cell samples: Initially, i.e. just after thawing and washing PBMCs in RPMI +
157 2% FCS, cells were incubated with 50 nM dasatinib, 30 min, 37 °C and resuspended in RPMI +
158 10% FCS to 0.2×10^6 cells ml^{-1} . Consecutive five-fold dilutions of PBMCs from one healthy donor
159 into PBMCs from another healthy donor generated seven samples with: 100%, 20%, 4%, 0.8%,
160 0.16%, 0.032% and 0.0064% cells derived from the first donor PBMCs. Cells were washed in
161 barcode-cytometry buffer and incubated with multimers and antibodies as described above.

162 Cells applied for tetramer staining were cryopreserved, thawed, washed and incubated with
163 dasatinib in the same way as cells stained with barcode-labeled multimers. Tetramer stainings were
164 performed as described previously^{4,12}.

165

166 **Intracellular cytokine staining**

167 Healthy donor PBMCs and TILs were thawed and washed in RPMI + 10% FCS. TILs were rested
168 overnight in X-vivo + 5% human serum at 37 °C and 5% CO₂, washed and re-suspended in X-vivo
169 + 5% human serum. 3x10⁵ TILs were stimulated with autologous tumor at a ratio 3:1 (TIL:tumor)
170 and 1μL/mL GolgiPlug (BD, 555029) was added prior to incubation, 5h, 37 °C and 5% CO₂. 1x10⁶
171 healthy donor PBMCs were stimulated with an extended CEF peptide pool (jpt, PM-CEF-E) at a
172 concentration of 1 μg/ml of each peptide. After 2h incubation, 37 °C and 5% CO₂, 1 μL/mL
173 GolgiPlug (BD, 555029) was added and cells were incubated 4 more hours at 37 °C and 5% CO₂.
174 TILs and healthy donor PBMCs were washed twice in barcode-cytometry buffer and incubated, 15
175 min, 37 °C, with pooled DNA-barcoded multimers in a total volume of 100 μL (final concentration
176 of each distinct pMHC, 23μM). Cells were stained with surface antibodies: FITC-conjugated anti-
177 CD3 antibody (BD 345763) and PerCP-conjugated anti-CD8 antibody (Invitrogen MHCD0831)
178 (final dilution 1/40 of each antibody) and dead cell marker (LIVE/DEAD Fixable Near-IR;
179 Invitrogen L10119) (final dilution 1/1000) and incubated for 30 min, 4 °C. Cells were washed twice
180 in barcode-cytometry buffer, incubated 30 min-O.N., 4 °C, in fixation buffer (1:4, eBioscience 00-
181 5123-43, to diluent eBioscience 00-5223-56), washed and resuspended in permeabilization buffer
182 (1:10 buffer to water, eBioscience 00-8333-56). Cells were stained with intracellular antibodies:
183 PE-Cy7-conjugated anti-TNFα antibody (BioLegend 502930) and APC-conjugated anti-IFNγ
184 antibody (BD 341117) (final dilution 1/10 of each antibody) and incubated 30 min, 4 °C. Cells were
185 washed in permeabilization buffer and resuspended in barcode-cytometry buffer. If cells were not
186 acquired immediately, they were resuspended in 1% PFA and washed twice more in barcode-
187 cytometry buffer after 1-24h. Fixed cells were acquired within a week after staining.

188

189 **Flow cytometry and cell sorting**

190 Cells stained with DNA-barcoded multimers were sorted on a FACSAria (Aria, Aria-II or
191 AriaFusion) (Becton Dickinson) into tubes containing 200 μ L barcode-cytometry buffer (tubes were
192 saturated with PBS + 2% BSA in advance). Using FACSDiva software we gated on single, live
193 CD8⁺ positive and ‘dump’ (CD4, 14, 16, 19 and 40) negative lymphocytes and sorted all multimer
194 (PE) positive cells within this population. For the samples stained with antibodies for intracellular
195 activation-markers and DNA-barcoded-multimers, we gated on single, live CD8⁺/CD3⁺
196 lymphocytes. In all ICS experiments we sorted the IFN- γ /TNF- α double positive and the double
197 negative population into separate tubes. The sorted cells were centrifuged 10 min, 5000 g, and the
198 buffer was removed. The cell pellet was stored at -80 °C in a minimal amount of residual buffer
199 (<20 μ L).

200 Tetramer stained cells were acquired on a LSR-II or a LSR-Fortessa flow cytometer (Becton
201 Dickinson). Antigen-specific T cells were identified as described in^{4,12}. Briefly, we gated on single,
202 live CD8⁺ positive and ‘dump’ (CD4, 14, 16, 19 and 40) negative lymphocytes and selected cells
203 positive in two tetramer channels and negative in the seven remaining tetramer channels.

204

205 **DNA barcode amplification**

206 Taq PCR Master Mix Kit (Qiagen, 201443) was used for amplification of DNA barcodes using 0.3
207 μ M of appropriate forward and reverse primers comprising Ion Torrent PGM 5’ and 3’ adaptors (A-
208 key and P1-key, respectively). PCR amplification was conducted on isolated cells (in <20 μ l of
209 buffer) or on the stored aliquot of the MHC multimer reagent pool (diluted 50,000x in the final
210 PCR) used as baseline to determine the number of DNA barcode reads within a non-processed
211 MHC multimer reagent library. Each sample was assigned a distinct sampleID embedded in the

212 forward primer. PCR was performed under the following conditions: 95 °C 10 min; 36 cycles: 95
213 °C 30 s, 60 °C 45 s, 72 °C 30 s; and 72 °C 4 min. The PCR products were purified with QIAquick
214 PCR Purification kit (Qiagen, 28104) or QIAquick gel extraction kit (Qiagen, 28704). The
215 amplified DNA barcodes were sequenced at Sequetech (U.S.A.) or GeneDx (U.S.A.) using Ion
216 Torrent PGM 314 or 316 chip (Life Technologies).

217

218 **Processing of sequencing data**

219 Raw sequence reads were aligned to the constant primer regions and the 16 nucleotide AxBy
220 annealing region using Bowtie2⁴⁷. Reads successfully mapped to at least two of these three features
221 were aligned to the primer regions, the 25mer barcode regions, and the annealing region using the
222 Smith-Waterman algorithm. Based on these alignment positions, the sampleID and the UMI (Fig
223 1B) were located and extracted. Sequence reads that could not be unambiguously assigned to a
224 DNA barcode were discarded. To avoid multiple counting of duplicate sequence reads generated by
225 amplification, the two random UMI regions were concatenated and duplicate counts for each AxBy
226 barcode combination were removed. The resulting $n \times m$ matrix of clonally unique sequence
227 counts, where n is the number of samples and m is the number of AxBy barcodes, was used in all
228 further analyses.

229

230 **Statistical analyses**

231 The analysis of barcode enrichment was based on methods designed for the analysis of RNA-seq
232 data and was implemented in the R package edgeR⁴⁸. Fold changes in read counts mapped to a
233 given sample relative to mean read counts mapped to triplicate baseline samples were estimated
234 using normalization factors determined by the trimmed mean of M-values method⁴⁹. P values were

235 calculated by comparing each experiment individually to the mean baseline sample reads using a
236 negative binomial distribution with a fixed dispersion parameter set to 0.1; this value was derived
237 from the background distribution in the 10 healthy donor PBMC samples shown in Figure 3. False
238 discovery rates (FDRs) were estimated using the Benjamini-Hochberg method. Specific barcodes
239 with an FDR<0.1% were defined as significant. At least 1/1000 reads associated with a given DNA
240 barcode relative to the total number of DNA barcode reads in that given sample was set as threshold
241 avoid false positive detection of T cell populations due to low number of reads in the baseline
242 samples.

243

244 **QPCR-based recovery of enriched DNA barcodes**

245 QPCR-based analyses were used to recover enriched barcodes when the number of distinct
246 barcodes in a given experiment where ≤ 2 . The Brilliant II QRT-PCR Low ROX Master Mix Kit
247 (Agilent 600837) was applied with 0.3 μ M 5' and 3' primers (forward primers:
248 **GAAGTTCCAGCCAGCGTC** or **GAGATACGTTGACCTCGTTG** and reverse primers:
249 **CTGTGACTATGTGAGGCTTTC** or **ATGCAACCAAGAGCTTAAGT**) and 0.1 μ M sequence
250 specific fluorescent reporter probes (probeB1: 6-
251 FAM/GCCTGTAGTCCCACGCGATCTAACA/BHQ and probeB2:
252 HEX/CAACCATTTGATTGGGGACAACCTGGG/BHQ or probe-ss1: 6-
253 FAM/TCT[+T][+G][+A]AC[+T][+A]TG[+A][+A][+T]CGTC/BHQ-1-plus and probe-ss2:
254 HEX/TCT[+A][+T][+A]GG[+T][+G]TC[+T][+A][+C]TACC/BHQ-1-plus) ([+X]= locked nucleic
255 acid). Template was drawn from residual buffer containing the sorted cells (≤ 20 μ L per sample). A
256 40-cycle qPCR (Mx3000P, Agilent) was performed with the following cycling conditions: 95 °C 10
257 min; 95 °C 30 s, 60 °C 60 s \times 40 cycles.

258

259 **Note on DNA-barcodes**

260 For the initial experiments presented in figure S1C we used single-stranded DNA barcodes (ss-
261 barcodes) that resembled the A-oligonucleotides, but where the annealing region at the 3' end on the
262 A-oligonucleotides were replaced with a reverse primer region as follows: ss-barcode1: **Biotin-**
263 **GAGATACGTTGACCTCGTTGAANNNNNN**TCTTGA ACTATGAATCGTCTCACTTAAGCTC
264 **TTGGTTGCAT** and ss-barcode2: **Biotin-**
265 **GAGATACGTTGACCTCGTTGAANNNNNN**TCTATAGGTGTCTACTACCTCACTTAAGCTC
266 **TTGGTTGCAT** (blue, green and black coloring indicates primer regions, UMI and the 25mer
267 barcode region, respectively).

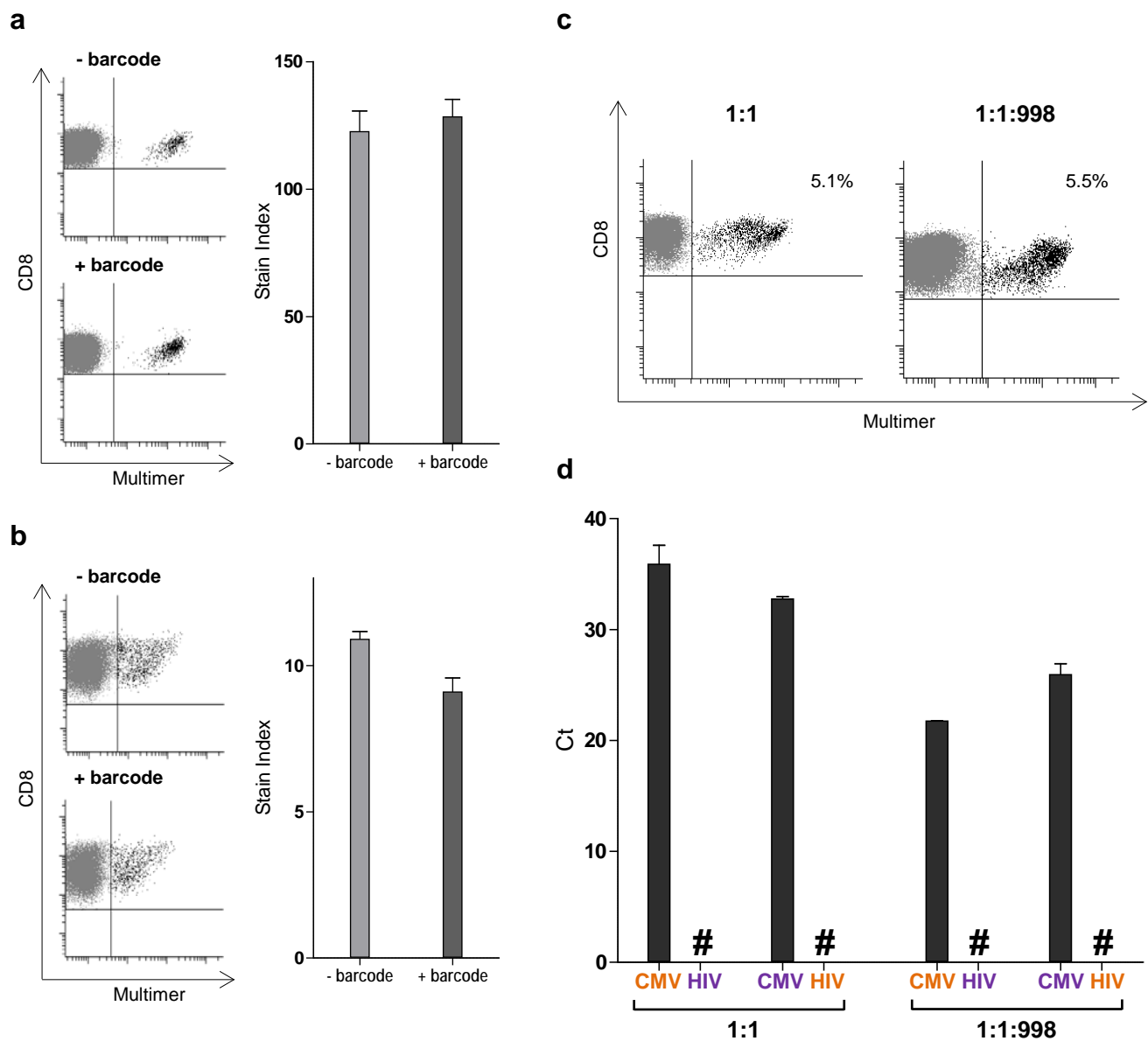
268

269 **Supplementary References**

- 270 32. Donia, M. *et al.* Characterization and comparison of 'standard' and 'young' tumour-
271 infiltrating lymphocytes for adoptive cell therapy at a danish translational research
272 institution. *Scand. J. Immunol.* **75**, 157–167 (2012).
- 273 33. Donia, M. *et al.* Aberrant Expression of MHC Class II in Melanoma Attracts Inflammatory
274 Tumor-Specific CD4⁺ T- Cells, Which Dampen CD8⁺ T-cell Antitumor Reactivity. *Cancer*
275 *Res.* **75**, 3747–59 (2015).
- 276 34. Gerlinger, M. *et al.* Intratumor heterogeneity and branched evolution revealed by multiregion
277 sequencing. *N. Engl. J. Med.* **366**, 883–92 (2012).
- 278 35. Gerlinger, M. *et al.* Genomic architecture and evolution of clear cell renal cell carcinomas
279 defined by multiregion sequencing. *Nat. Genet.* **46**, 225–33 (2014).

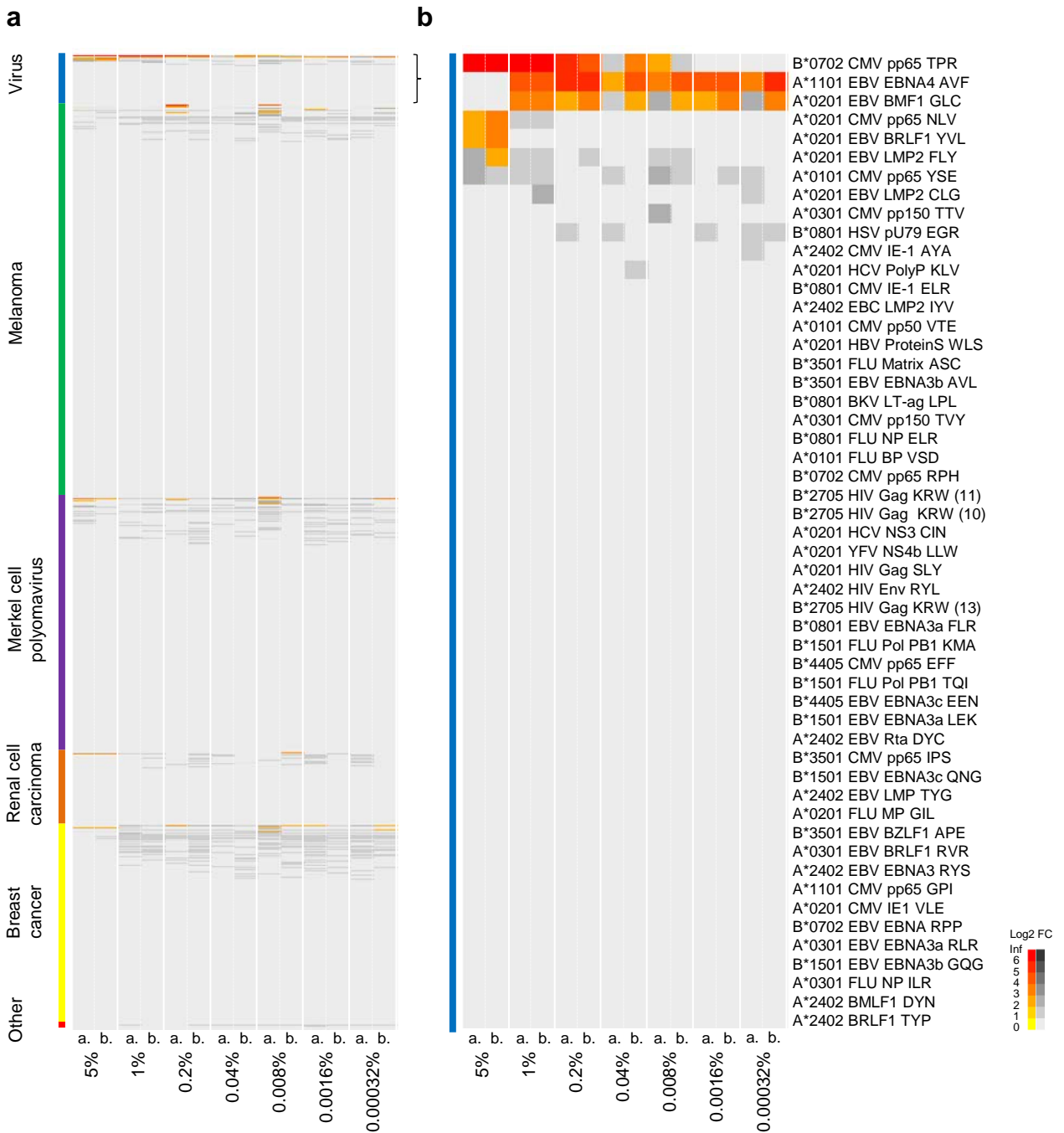
- 280 36. Szolek, A. *et al.* OptiType: precision HLA typing from next-generation sequencing data.
281 *Bioinformatics* **30**, 1–7 (2014).
- 282 37. Bakker, A. H. *et al.* Conditional MHC class I ligands and peptide exchange technology for
283 the human MHC gene products HLA-A1, -A3, -A11, and -B7. *Proc.Natl.Acad.Sci.U.S.A*
284 **105**, 3825–3830 (2008).
- 285 38. Rodenko, B. *et al.* Generation of peptide-MHC class I complexes through UV-mediated
286 ligand exchange. *Nat.Protoc.* **1**, 1120–1132 (2006).
- 287 39. Toebes, M. *et al.* Design and use of conditional MHC class I ligands. *Nat.Med.* **12**, 246–251
288 (2006).
- 289 40. Hadrup, S. R. *et al.* High-throughput T-cell epitope discovery through MHC peptide
290 exchange. *Methods Mol. Biol.* **524**, 383–405 (2009).
- 291 42. Chang, C. X. L. *et al.* Conditional ligands for Asian HLA variants facilitate the definition of
292 CD8+ T-cell responses in acute and chronic viral diseases. *Eur. J. Immunol.* **43**, 1109–20
293 (2013).
- 294 43. Garboczi, D. N., Hung, D. T. & Wiley, D. C. HLA-A2-peptide complexes: refolding and
295 crystallization of molecules expressed in *Escherichia coli* and complexed with single
296 antigenic peptides. *Proc.Natl.Acad.Sci.U.S.A* **89**, 3429–3433 (1992).
- 297 44. Hoof, I. *et al.* NetMHCpan, a method for MHC class I binding prediction beyond humans.
298 *Immunogenetics* **61**, 1–13 (2009).
- 299 45. Nielsen, M. *et al.* NetMHCpan, a method for quantitative predictions of peptide binding to
300 any HLA-A and -B locus protein of known sequence. *PLoS.One.* **2**, e796 (2007).
- 301 46. Rammensee, H. G., Bachmann, J., Emmerich, N. P., Bachor, O. A. & Stevanovic, S.

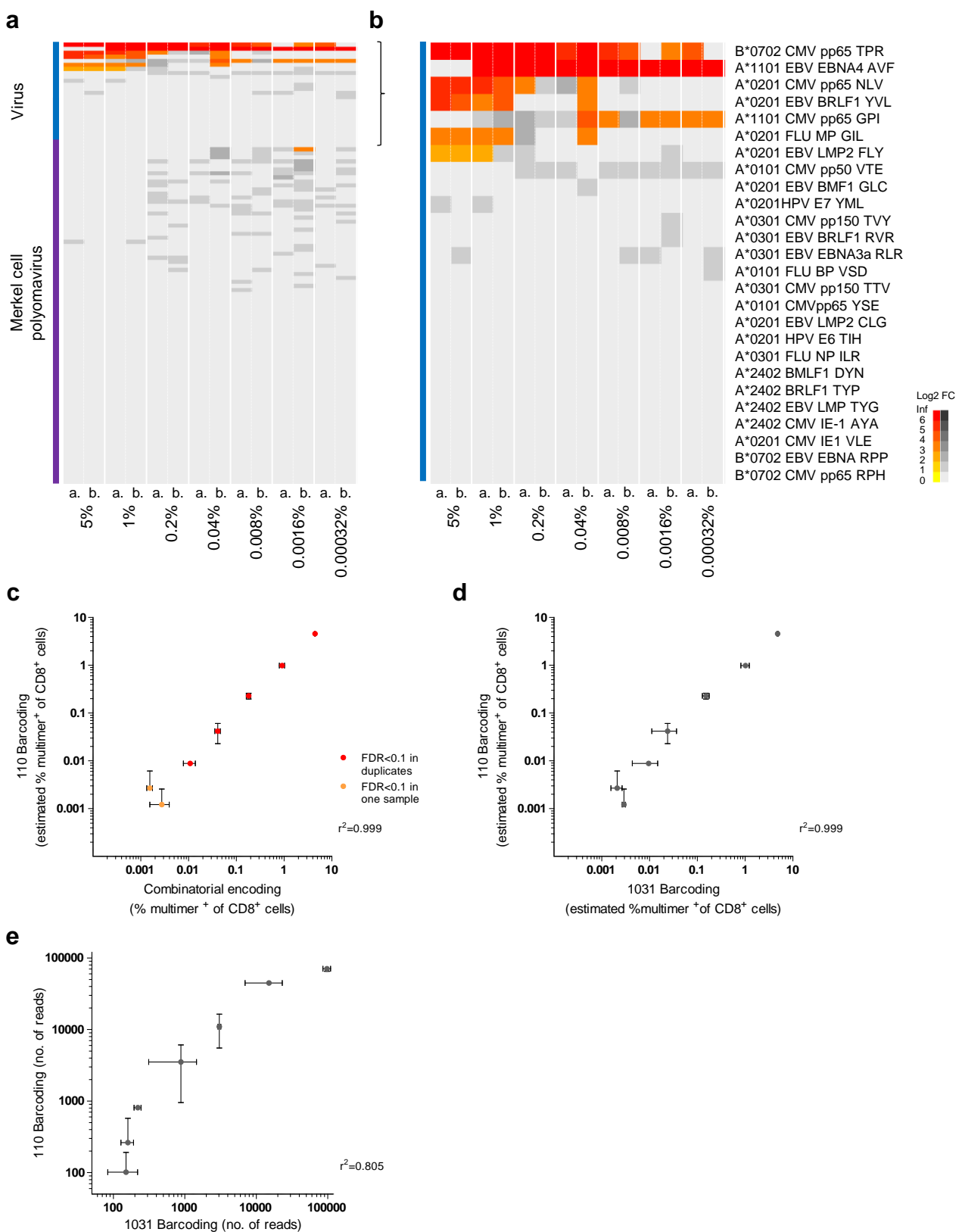
- 302 SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* **50**, 213–219
303 (1999).
- 304 47. Langmead, B. & Salzberg, S. L. Fast gapped-read alignment with Bowtie 2. *Nat. Methods* **9**,
305 357–9 (2012).
- 306 48. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for
307 differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–40
308 (2010).
- 309 49. Robinson, M. D. & Oshlack, A. A scaling normalization method for differential expression
310 analysis of RNA-seq data. *Genome Biol.* **11**, R25 (2010).
- 311



Supplementary Figure 1. Binding capacity of DNA-barcoded MHC multimers and recovery of antigen specificity.

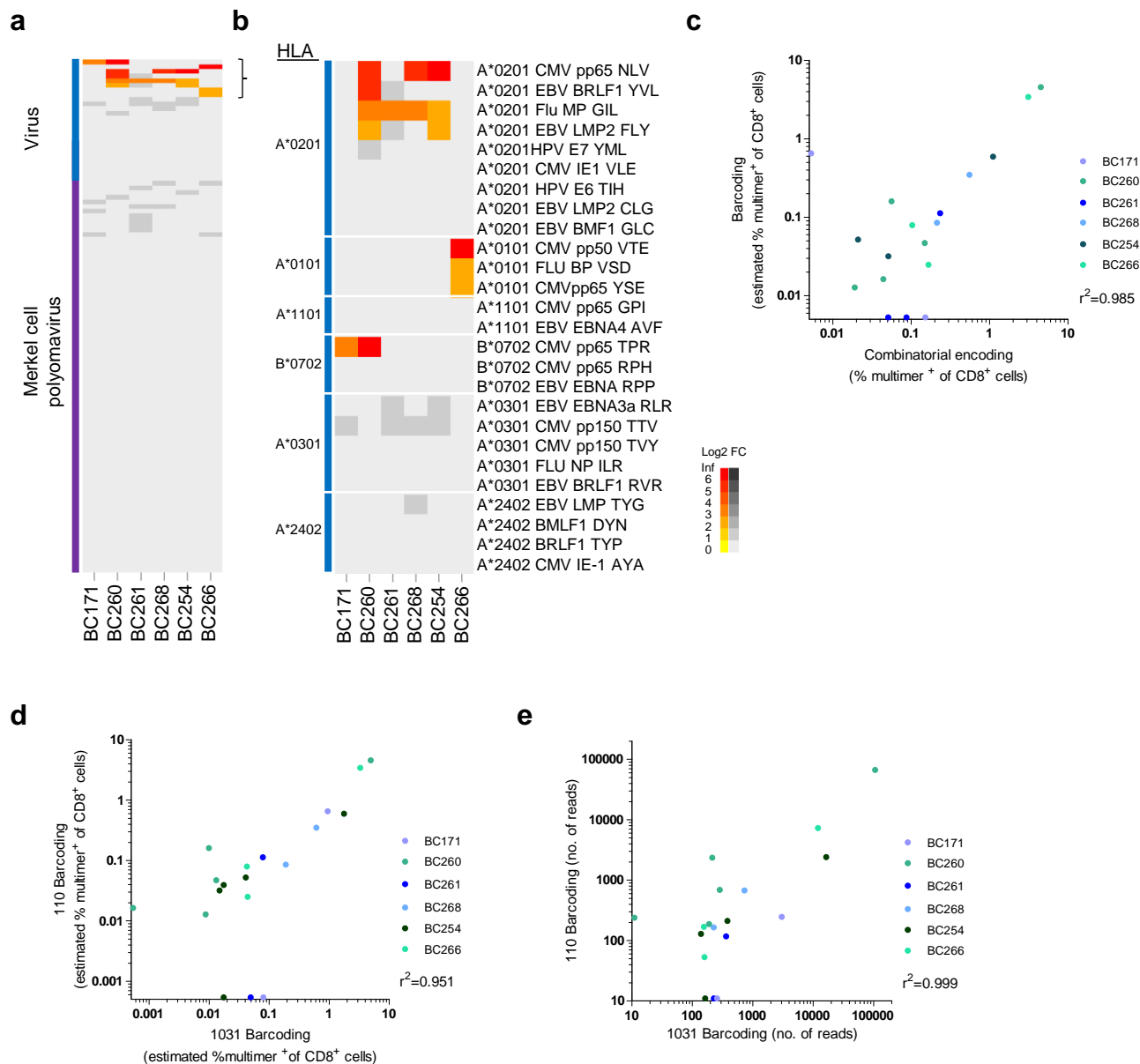
(a, b) Fluorescent-based determination of the binding capacity of DNA-barcoded MHC multimers (+barcode) compared to fluorescent labeled MHC multimers (-barcode). DNA-barcoded MHC multimer reagents were assembled with two identical DNA barcodes attached to each multimerization backbone and subsequent co-attachment of pMHC molecules. Non-barcoded multimers were generated similarly without prior attachment of DNA barcodes (i.e. '-barcode' and '+barcode' were both assembled on a dextramer backbone). Reagents assembled with HLA-A*0201 CMV pp65_{NLV} were applied for staining (a) of healthy donor PBMCs and reagents assembled with HLA-A*0201 hTERT p988 (YLQVNSLQTV) were applied for staining of (b) expanded TILs from a melanoma patient. The binding capacity is evaluated in terms of the stain index of the multimer fluorescent intensity of T cells stained with non-barcoded or DNA-barcoded MHC multimers respectively, along with the frequencies of the given multimer positive cell population of CD8 T cells, (a) 0.9-1.2% and (b) 3.8%-4.3%. Bar plots show mean stain index values of three stainings \pm SD. (c) Fluorescent-based analysis of antigen specific T cells stained with relevant virus pMHC multimers and excess of irrelevant pMHC multimers. An equimolar (1:1) mixture of individually barcode labeled HLA-B*0702 CMV pp65_{T_{PR}}-multimers and HLA-A*0201 HIV Pol_{ILK}-multimers, or a mixture with further 998 additional of fluorescent labeled pMHC multimers, were used for staining of healthy donor PBMCs. The 998 additional MHC multimers comprised equal amounts of irrelevant-peptide HLA-A*0201 and HLA-B*0702 multimers, i.e. multimers carrying MHC molecules refolded with UV-sensitive ligand (Supplementary Methods). Using either reagent mixture the concentrations of each equimolar pMHC were 23 μ M in the final staining volume, i.e. for staining with the 1:1:998 equimolar reagents, the volume of the MHC multimer pool were reduced 50x. Percentages of the multimer positive population of CD8 T cells are given in dot plots. (d) The multimer positive populations from (c) were sorted by FACS and DNA barcodes associated with the sorted cell population were subjected to qPCR with fluorescent reporter probes targeting each individual DNA barcode. The experiments were performed with reagent mixtures with DNA barcodes inverted between the CMV and HIV pMHC multimers (indicated in orange and purple respectively). Cross threshold (Ct) values of DNA barcodes recovered from qPCR with approximately 200 and 600 cells in separate reactions (derived from staining with 1:1 and 1:1:998 reagent mixtures respectively) are shown in bar plots (mean \pm range of duplicate samples). Hashtag indicate that a given barcode was not detected.





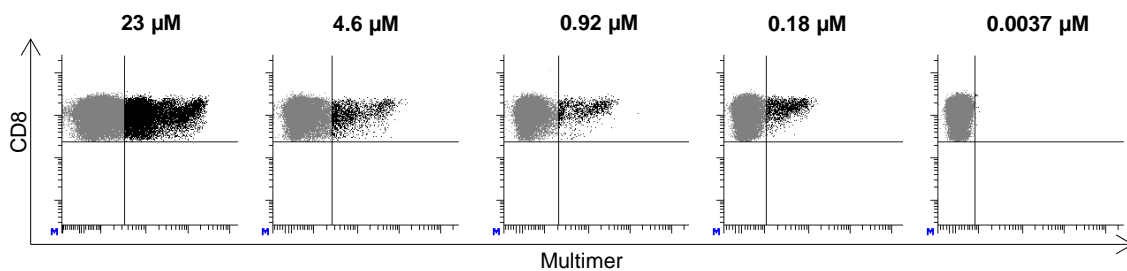
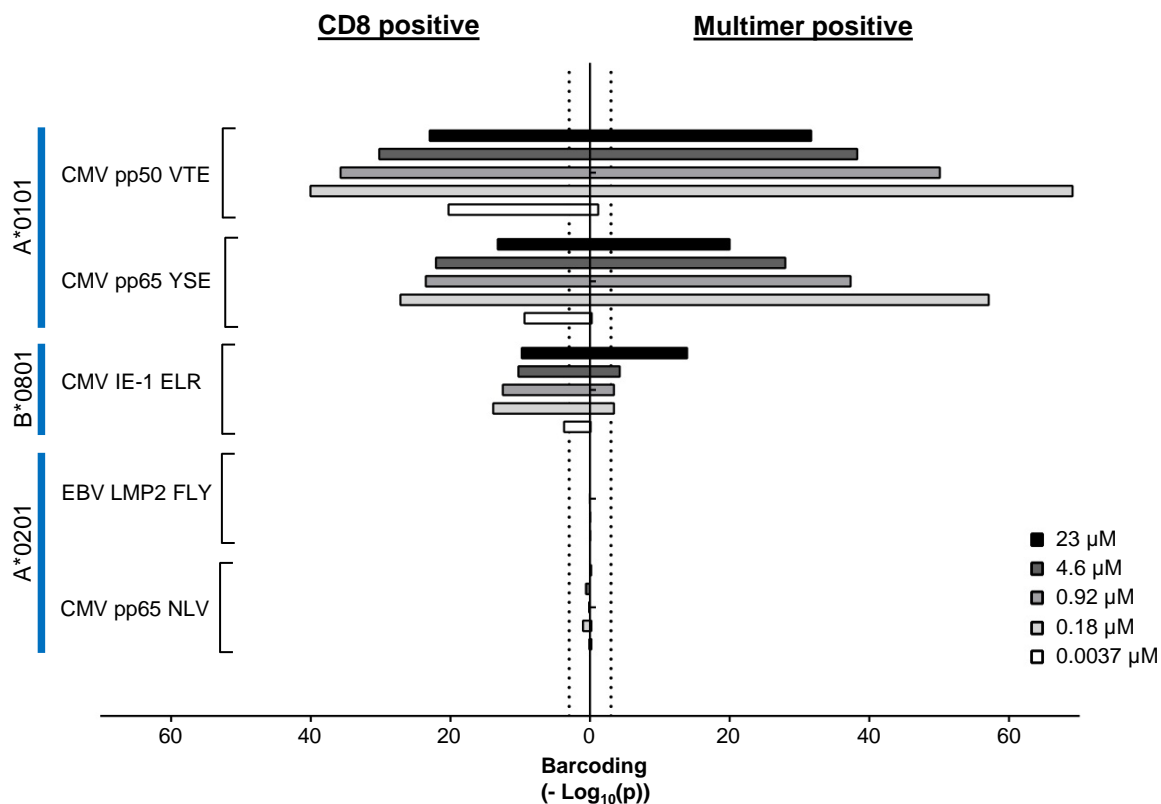
Supplementary Figure 3. Comparison of the dynamic range and detection limit using panels of 110 DNA-barcoded pMHC multimers or 1031 DNA-barcoded pMHC multimers. Related to Figure 2

(a) Heatmap representing the pMHC multimer analysis of duplicates of seven samples with various proportions of HLA-B*0702 CMV_{TPR}-specific T cells: 5%, 1%, 0.2%, 0.04%, 0.008%, 0.0016% and 0.00032% of CD8 T cells. The '5%' sample corresponds to 100% BC260 PBMCs. Each sample was screened with a panel of 110 pMHC multimers, all carrying individual DNA barcodes. The heatmap is organized as in Figure 2b, each column represents one donor. (b) Magnification of the panel of virus-derived peptides. Rows representing antigen specificities are sorted based on LogFC and duplicate samples a grouped as in (a). The first row represents the target '5%' titrated specificity, B*0702 CMV_{TPR}. The rows below are T cell responses present in the HLA-B*0702 negative PBMC sample (BC262) or lower-frequent responses present in the "5%" donor (BC260). Dark gray scaling, i.e. barcode reads that are non-significant but with LogFC > 1, may represent T cell responses just below the detection limit. (c) Correlations between the frequency of HLA-B*0702 CMV_{TPR}-specific T cells determined by analyzing the same samples using combinatorial fluorescent labeled pMHC multimers or a panel of 110 DNA barcode labeled pMHC multimers. (d, e) Correlation between results obtained from screening the same samples of 2x10⁶ PBMCs with the panel of 1031 or 110 DNA-barcoded pMHC multimers represented as (d) the estimated frequency of HLA-B*0702 CMV_{TPR}-specific T cells or (e) the number of clonally reduced barcode reads associated with this pMHC multimer. Error bars represent range of duplicates. The accumulated number of non-reduced read counts that mapped to any of the DNA barcodes among the 14 samples screened were 2.7x10⁶ and 1.4 x10⁶ reads for the 110 and 1031 pMHC multimer library respectively. All pMHC multimers are 'dextramers'.



Supplementary Figure 4. Assessment of T cell reactivity across healthy donor samples using a panel of 110 DNA-barcoded pMHC multimers. Related to Figure 3

(a) Analysis of PBMCs ($1-2 \times 10^6$) from six different healthy donors using 110 pMHC multimers each carrying individual barcodes. The heatmap is organized as in figure 2B, each column represents one donor. Epitopes are grouped based on their antigen origin. Significant responses are shown in yellow-red colors. Significance was defined as $FDR < 5\%$ since the number of reads within a given sample were compared with only one baseline sample (opposed to three baseline samples in other experiments). (b) Magnification of the panel of virus-derived peptides (26 epitopes). Rows representing antigen specificities are grouped according to HLA-type and sorted within each group based on Log_2FC . HLA types of donors can be seen in table S8. (c) Correlations between the frequencies of antigen-specific T cells determined by analyzing the same samples with combinatorial encoded fluorescently labeled pMHC multimers or with 110 DNA-barcoded pMHC multimers. Each dot represents one specificity. T cell populations with $FDR < 5\%$ in DNA-barcode MHC multimer analysis or ≥ 10 events and $> 0.002\%$ of CD8 T cells in combinatorial encoding analysis were plotted. All specificities included in the plot were tested using both a combinatorial encoding analysis and DNA-barcoded MHC multimers. Dots plotted on the axes are nonsignificant for one of the methods. (d, e) Correlation between results obtained from screening the same samples with the panel of 1031 or 110 DNA-barcoded pMHC multimers represented as (d) the estimated frequency of antigen-specific T cells or (e) the number of clonally reduced barcode reads associated with the given pMHC multimers. Each dot represents one specificity. Only T cell populations that fulfilled the significance criteria for DNA barcode assessment ($FDR < 0.1\%$ and 5% for the 1031 and 110 library, respectively) in at least one of the analyses were plotted. Dots plotted on the axes are nonsignificant for one of the library screenings. The accumulated number of non-reduced read counts that mapped to any of the DNA barcodes among the six screened samples were 2.6×10^5 and 4.3×10^5 reads for the 110 and 1031 pMHC multimer library respectively.

a**Multimer concentration:****b**

Supplementary Figure 5. T cell reactivity assessed independently from fluorescent-based separation of MHC multimer binding T cells. Related to Figure 4

(a) PBMCs (2×10^6) from one healthy donor was stained with varying amounts of DNA barcoded-pMHC multimers, i.e. a titration from $23 \mu\text{M}$ to $0.0037 \mu\text{M}$ in respect to each pMHC as indicated above each dot plot. This corresponds to 100%-0.016% of the amount used elsewhere in this study. Samples were stained in duplicates and either the full CD8 population (all cells in the dot plots) or only the MHC multimer positive population (cells indicated in black) were sorted. (b) Bar plot representing the distribution of DNA barcodes in the isolated cells from (a). Left side is based on the full CD8 population, right side is based of the MHC multimer positive population. Each bar represents the $-\text{Log}_{10}(p)$ value in respect to the pMHC associated DNA barcode. Dotted line at $y=3$ ($-\text{Log}_{10}(0.001)$) represent the threshold of $\text{FDR} < 0.1\%$. The donor BC035 is HLA-A*0101 and B*0801 positive and A*0201 negative (see full HLA-type in Supplementary Table 7). BC035 was previously found to carry antigen-responsive T cells restricted to HLA-A*0101, CMV pp50_{VTE} (2.2% of CD8 T cells) and CMV pp65_{YSE} (0.6% of CD8 T cells). HLA- A*0201 restricted epitopes functions as negative controls. Irrespectively of sorting the full CD8 or only the multimer positive population the same responses are detected after sequencing of the DNA barcodes. When the MHC multimer binding T cells could not be separated from the full CD8 T cell population based on there fluorescent intensity, i.e. when applying the lowest amount of MHC multimer reagent, the DNA barcodes associated with positive control reagents were still recovered after sorting the full CD8 population indicating that T cell reactivity can be assessed independent on fluorescent separation of the MHC multimer binding T cells.