BATF–JUN is critical for IRF4–mediated transcription in T cells

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Interferon regulatory factor 4 (IRF4) is an IRF family transcription factor with critical roles in lymphoid development and in regulating the immune response2,3. IRF4 binds DNA weakly owing to a carboxy-terminal auto-inhibitory domain, but cooperative binding with factors such as PU.1 or SPIB in B cells increases binding affinity4, allowing IRF4 to regulate genes containing ETS–IRF composite elements (EICEs; 5’-GGAAnGAAA-3’). Here we show that in mouse CD4+ T cells, where PU.1/SPiB expression is low, and in B cells, where PU.1 is well expressed, IRF4 unexpectedly can cooperate with activator protein-1 (AP1) complexes to bind to AP1–IRF4 composite (5’-TGAnTCA/GAAA-3’) motifs that we denote as AP1–IRF composite elements (AICEs). Moreover, BATF–JUN family protein complexes cooperate with IRF4 in binding to AICEs in pre-activated CD4+ T cells stimulated with IL-21 and in T(H)17 differentiated cells. Importantly, BATF binding was diminished in IRF4+ T cells and IRF4 binding was diminished in BATF–JUN T cells, consistent with functional cooperation between these factors. Moreover, we show that AP1 and IRF complexes cooperatively promote transcription of the Il10 gene, which is expressed in T(H)17 cells and potently regulated by IL-21. These findings reveal that IRF4 can signal via complexes containing ETS or AP1 motifs depending on the cellular context, thus indicating new approaches for modulating IRF4-dependent transcription.

There are nine mammalian interferon regulatory factor (IRF) family members, IRF1 to IRF9, that collectively have broad actions within and beyond the immune system2. IRFs were identified on the basis of their expression in cells beyond the immune system2. IRFs were identified on the basis of their members, IRF1 to IRF91, that collectively have broad actions within and beyond the immune system2,3. IRFs were identified on the basis of their induction by type I interferons (IFN-α/β), and some IRFs are induced by Toll-like receptors and other pattern-recognition receptors1,2. IRF4 expression is restricted to the immune system and is induced in T cells by T-cell receptor stimulation5,6. IRFs contain an amino-terminal DNA-binding domain that recognizes 5’-GGAAnGAAA-3’ motifs, but IRF4 only weakly binds DNA due to its carboxy-terminal auto-inhibitory domain5. In B cells, PU.1 or the related factor SPIB relieves auto-inhibition to increase binding affinity, allowing IRF4 to regulate genes expressing composite 5’-GGAAnGAAA-3’ ETS–IRF consensus motif elements (EICEs)6,9, including γ and λ immunoglobulin light chain genes. Whereas PU.1 binds directly to EICEs, efficient IRF4 binding requires phosphorylated, DNA-bound PU.11,2. IRF4 also acts in T cells6, contributing to development of multiple T(H)1 cell subsets5, with defective T(H)1, T(H)2, T(H)9 and T(H)17 differentiation in its absence. Using genome-wide chromatin immunoprecipitation coupled to DNA sequencing (ChIP-Seq), we previously demonstrated that IRF4 cooperates with STAT3 to control IL-21–induced Prdm1 expression and that these factors globally regulate IL-21–mediated gene expression6. Moreover, we found that IRF4 expression is required for normal STAT3 binding in vivo and for development of an additional T-cell effector population, namely Th8 cells6.

As anticipated, analysis of IRF4 ChIP-Seq peaks from B cell libraries we previously generated14 identified EICEs as the top motif (Fig. 1a, b). In contrast, EICEs were not readily identified in IRF4 ChIP-Seq libraries from activated T cells (Fig. 1c, d) or T(H)17 cells (Fig. 1e), consistent with T cells expressing much less PU.1 than B cells (Fig. 1f and Supplementary Fig. 1). Instead, examination of the top 1,000 peaks (sorted by P values) from libraries from pre-activated T cells, unstimulated or stimulated with IL-21, or from T(H)17 cells, unexpectedly revealed that the top IRF4 ChIP-Seq motifs were activator protein-1 (AP1) 5’-TGAnTCA/GAAA-3’ motifs (Fig. 1c–e). IRF8, which like IRF4 also interacts with PU.16, bound to some

Figure 1 | Preferential IRF4 binding to AP1 motifs in T cells. a–e, IRF4 motifs in B cells not stimulated (a) or stimulated (b) with IL-21, in pre-activated T cells not stimulated (c) or stimulated (d) with IL-21 for 1 h, or in T(H)17 cells (e). f, Sfpi1/PU.1 mRNA expression in the indicated populations based on RNA-Seq. g, 5’-TTCC/GAAA-3’ motif distribution relative to the 5’-TGAnTCA/GAAA-3’ AP1 motif in 1000 sequences with strong IRF4 binding, and the number of IRF4 ChIP-Seq peaks at each spacing. The blue line shows the 5’-TTCC/GAAA-3’ distribution in 1000 random sequences; motif frequency was approximately 0.5%.

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AP1-containing sites but dominantly bound to canonical IRF motifs with tandem GAAA motifs (5'-GAAA[C/G][T/A]GAAA[G/C]-3') (Supplementary Fig. 2a, b). To elucidate the IRF4–AP1 interaction, we examined whether IRF4 GAAA/TTTC core motifs were associated with AP1 sites, and within IRF4 ChIP-Seq peaks, we found enrichment of these motifs adjacent to or five base pairs away (four intervening base pairs) from AP1 sites (Fig. 1g and Supplementary Fig. 3), in contrast to their overall random distribution (Fig. 1g, blue line), indicating binding cooperativity for AP1 and IRF4. We denote these AP1–IRF4 composite elements as AICEs.

We compared IRF4 ChIP-Seq peaks with the DNA motifs associated with AP1 sites in TH17 cells, and found 14,838 IRF4 ChIP-Seq peaks, 5,304 bound within genes annotated by RefSeq (Fig. 2a), and analysis of our published Affymetrix array data sets revealed that 2,356 of these genes were regulated by IL-21 at 1, 6 or 24 h (Fig. 2b). RNA-Seq analysis revealed markedly lower expression of some of these genes, including Prdm1 and Il10, in IL-21-stimulated Irif4−/− than in wild-type T cells (Fig. 2c), underscoring the importance of IRF4 for their expression. To characterize the IRF4-binding complex in T cells, we analysed ChIP-Seq libraries from Th17-differentiated cells and IL-21-stimulated pre-activated T cells, focusing on JUN family proteins and BATF, which can heterodimerize with JUN proteins to bind to AP1 motifs and is critical for Th17 differentiation15–17, a process promoted by IL-2118–20.

In TH17 cells, approximately 54% (11,693 out of 21,775) of the IRF4 motifs adjacent to or five base pairs away from AP1 sites, and within IRF4 ChIP-Seq peaks, we found enrichment of these motifs adjacent to or five base pairs away (four intervening base pairs) from AP1 sites (Fig. 1g and Supplementary Fig. 3), in contrast to their overall random distribution (Fig. 1g, blue line), indicating binding cooperativity for AP1 and IRF4. We denote these AP1–IRF4 composite elements as AICEs.

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supershifting was with anti-IRF4, anti-JUND, or anti-BATF. EMSA were with nuclear extracts from 293T cells transfected with IRF4, JUND, and BATF; revealed that IRF4, JUNB, JUND and BATF were components of the AP1 motif was mutated (Fig. 3b). Supershifting with antibodies against IRF4, BATF and JUNB, but not PU.1 (Supplementary Fig. 7a), even though anti-PU.1 antibody supershifted a complex formed with an EICE probe from the immunoglobin λ light chain enhancer (Supplementary Fig. 7b). Thus, EICEs were the most common IRF4-containing complexes in B cells (Fig. 1 a, b), but IRF4/AP1 AICEs also reported to express PU.1 protein, RNA-Seq analysis showed little PU.1 messenger RNA in these cells (Fig. 1f), and EMSAs showed IRF4–BATF–JUNB interactions but no PU.1-binding activity (Supplementary Fig. 7c). To determine whether IRF4 and BATF–JUN proteins cooperatively bound to DNA, we used nuclear extracts from 293T cells transfected with various combinations of IRF4, BATF, and JUNB or JUND and performed EMSAs with Il10, Cldn4 and Ikek2 probes. Little if any binding activity was observed with extracts from 293T cells expressing IRF4, JUND or BATF alone, certain pairwise combinations showed some binding, but strong binding was seen with extracts containing all three proteins, indicating cooperative binding to these sites (Fig. 3d, left); this was also observed when JUND was substituted for JUND (Fig. 3d, right). Cooperative binding was indicated by slower mobility, particularly of the Il10 peak 1 probe (Fig. 3d). Although mobility changes for other probes were less evident, even on 4% or 7% gels (not shown), supershifting experiments confirmed that IRF4, JUND and BATF were present in complexes formed with each probe (Fig. 3e).

To examine the functional significance of the Il10 IRF4 motif, we first analysed Il10 mRNA expression in Irf4−/− T cells and found much lower Il10 mRNA in response to IL-21, anti–CD3 + anti–CD28, or IL-21 + anti–CD3 + anti–CD28 than was observed in wild-type cells (P < 0.02 at both time points; Fig. 4a). Correspondingly, Il10 luciferase reporter activity was potently induced by IL-21 or anti–CD3 + anti–CD28, and more so by IL-21 + anti–CD3 + anti–CD28, but expression was diminished when the GAAAT motif or associated AP1 site was mutated (P < 0.02; Fig. 4b). Moreover, IRF4, JUND, JUNB and BATF probes (Fig. 3c). As expected, no shift was seen when nuclear extracts were omitted (Supplementary Fig. 6). Antibodies to FOS and FRA2 had a minor effect on the Il10 peak 1 and no effect on the Il10 peak 2 and Ikek2 complexes (Fig. 3c). In contrast, an AP1 consensus probe complex was not supershifted by anti-IRF4 but was supershifted by antibodies against BATF, FOS and FRA2 (Fig. 3c). We next studied binding to the Il10 peak 1 IRF4 motif in B cells, T2 and T9 cells, which all express Il10. B-cell nuclear extracts formed a complex supershifted by antibodies against IRF4, BATF and JUNB, but not PU.1 (Supplementary Fig. 7a), even though anti-PU.1 antibody supershifted a complex formed with an EICE probe from the immunoglobin λ light chain enhancer (Supplementary Fig. 7b). Thus, EICEs were the most common IRF4-containing complexes in B cells (Fig. 1 a, b), but IRF4/AP1 AICEs also reported to express PU.1 protein, RNA-Seq analysis showed little PU.1 messenger RNA in these cells (Fig. 1f), and EMSAs showed IRF4–BATF–JUNB interactions but no PU.1-binding activity (Supplementary Fig. 7c). To determine whether IRF4 and BATF–JUN proteins cooperatively bound to DNA, we used nuclear extracts from 293T cells transfected with various combinations of IRF4, BATF, and JUNB or JUND and performed EMSAs with Il10, Cldn4 and Ikek2 probes. Little if any binding activity was observed with extracts from 293T cells expressing IRF4, JUND or BATF alone, certain pairwise combinations showed some binding, but strong binding was seen with extracts containing all three proteins, indicating cooperative binding to these sites (Fig. 3d, left); this was also observed when JUND was substituted for JUND (Fig. 3d, right). Cooperative binding was indicated by slower mobility, particularly of the Il10 peak 1 probe (Fig. 3d). Although mobility changes for other probes were less evident, even on 4% or 7% gels (not shown), supershifting experiments confirmed that IRF4, JUND and BATF were present in complexes formed with each probe (Fig. 3e).

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**Figure 3 | Cooperative IRF4/BATF/JUN binding.** a, Probes for IRF4 ChIP-Seq peaks in Il10, Ikek2, and Cldn4, and an AP1 probe. AP1 motifs are underlined. The AP1 probe is from ref. 15. b, EMSA with Il10 peak 1 probe (WT or IRF, or IRF/AP1 double mutants) and T917 nuclear extracts. c, EMSAs using Il10 peak 1, Ikek2, Ikek2, or AP1 probes and T917 nuclear extracts; supershifting was performed as indicated. Ctrl Ig, control immunoglobulin. d, EMSAs using nuclear extracts from 293T cells transfected with indicated cDNAs; T917 extract vs. no extract are also shown (10th vs. 9th lane). e, EMSAs with nuclear extracts from 293T cells transfected with IRF4, JUND, and BATF; supershifting was with anti-IRF4, anti-JUND, or anti-BATF. EMSA were performed at least 3 times.

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**Figure 4| Cooperative action of IRF4, BATF, and JUN on Il10 mRNA expression relative to Rpl7 in WT or Irf4−/− T cells pre-activated and treated as indicated for 4 or 24 h. (n = 3; mean ± s.d.).** a, WT or mutant Il10 reporter constructs transfected into pre-activated T cells and treated as indicated for 7 h (n = 3; mean ± S.D.). RLU, relative light units. c, EMSA with Il10 peak 1 probe and nuclear extracts from WT or Irf4−/− T cells stimulated with IL-21 for 4 h. Supershifting antibodies are indicated. EMSA was performed twice. d, IRF4, BATF, and pan-JUN ChIP-Seq data from WT or Irf4−/− T cells pre-activated and stimulated with IL-21 at the Il10 gene. e, IRF4 binding in WT vs. Batf−/− T cells (upper) and BATF binding in WT vs. Irf4−/− cells (lower). TSS, transcription start site. f, As in d, except at Il17ra.
each bound to a wild-type probe spanning this region, but binding was diminished when Irf4−/− nuclear extracts were used (Fig. 4c). Moreover, in ChIP-Seq experiments, there was markedly decreased binding of Irf4 in Batf−/− T cells and of Batf and JUN in Irf4−/− T cells (Fig. 4d) at the Il10 locus but also globally (Fig. 4e), including for example at the Il17a gene (Fig. 4f), consistent with defective Il17a expression and Tq17 differentiation in Irf4−/− and Batf−/−T cells. These results indicate cooperative binding and transcriptional activation by Irf4 and Batf–JUN family proteins.

Irf4 is a pleiotropic IRF family transcription factor with broad immunological actions. Its critical role in regulating immunoglobulin genes involves functional cooperation with the largely B-cell-restricted factor Pu.1. We now demonstrate that in T cells, where Pu.1 expression is low, Irf4 instead functionally cooperates with Ap1 family proteins to act via Aicebs, with functional cooperation with Batf and JUN family proteins in pre-activated T cells stimulated with IL-21 as well as in Tq2, Tq9 and Tq17 polarized cells. Interestingly, a number of genes we selected for analysis (Il10, Cite4, Il17a, Pdmd1 and Ifk2f) were functionally grouped in a study of Tq2 inhibitory effector cells during chronic inflammation as preferentially expressed in Il-10−/− versus Il-10−/− cells; it will be interesting to determine whether Irf4–Ap1-dependent gene expression helps to explain these observations. Although Irf4 and Batf cooperatively bound in the context of Aicebs, it was unclear if they expression helps to explain these observations. Although Irf4 and Batf, it will be interesting to determine whether Irf4–Ap1-dependent gene expression helps to explain these observations. Although Irf4 and Batf cooperatively bound in the context of Aicebs, it was unclear if they associated in the absence of these sites. In T cells, we could co-precipitate Batf and Jun (Supplementary Fig. 8), but we only co-precipitated Irf4 and Jun in a single experiment and could not co-precipitate Batf and Irf4. Thus, if a direct interaction occurs, it may be relatively weak, but the marked decrease of Batf binding in Irf4−/− and of Irf4 in Batf−/− cells (Fig. 4) indicates cooperative binding to Aicebs. This binding is dependent on the Batf leucine zipper domain26. The ability of Irf4 to act via two types of complexes, Pu.1–Irf4 Eicebs in B cells and Ap1–Irf4 Aicebs in T cells and to some degree in B cells, highlights mechanisms for Irf4-mediated transcriptional activation. The identification of the Irf4–Ap1 connection suggests new approaches may be employed to selectively target certain actions of Irf4, potentially allowing ways to manipulate the immune response in a cell-type-restricted fashion.

METHODS SUMMARY

T and B cells were cultured in standard medium. Cells were pre-activated with anti-CD3 + anti-CD28 for 3 days, rested overnight and stimulated with IL-21 for 1 h (ChIP-Seq) or 4 h (EMSA). Tq17 polarization was performed by standard methods and unlike Cd4+ T cells, Tq17 polarized cells were not stimulated with IL-21. With Batf−/− and Irf4−/− mice were 6–8 weeks old. C57BL/6 background mice of mixed gender. All experiments with mice were performed under protocols approved by the NHLBI Animal Care and Use Committee, and followed NIH guidelines for use of animals in intramural research. ChIP-Seq27 and RNA-Seq28 experiments were performed as previously described. ChIP-Seq and RNA-Seq libraries are summarized in Supplementary Table 2; details of binding site identification and motif analysis are in the online methods. For reporter assays, acti-vated T cells were electroporated with reporter plasmid and pRLTK, and dual luciferase assays performed. For EMSAs, nuclear extracts29 were bound to 32P-labelled probes. For supershifts, extracts were pre-incubated with indicated antibodies, before analysis on 5% polyacrylamide gels.

Full Methods and any associated references are available in the online version of the paper.

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1. Taniguchi, T., Ogasawara, K., Takaoka, A. & Tanaka, N. Irf family of transcription factors as regulators of host defense. Annu. Rev. Immunol. 19, 623–655 (2001).
2. Koh Moff, M. & Man, T. W. Roles of interferon-regulatory factors in T-helper-cell differentiation. Nature Rev. Immunol. 5, 125–135 (2005).
3. Brass, A. L., Kehrl, E., Eisenberg, C. F., Storb, U. & Singh, H. Pip, a lymphoid-restricted IRF, contains a regulatory domain that is important for autoimmunization and tertiary complex formation with the Ets factor Pu.1. Genes Dev. 10, 2335–2347 (1996).
4. Grossman, A. et al. Cloning of human lymphocyte-specific interferon regulatory factor (HLSIRF/HIRF) and mapping of the gene to 6p23–p25. Genomics 37, 229–233 (1996).
METHODS

Cells and cell culture. Mouse T and B cells were isolated using kits (Miltenyi) and cultured in RPMI-1640 medium containing 10% fetal bovine serum. Cells were pre-activated with plate-bound anti-CD3 (2 μg ml⁻¹) + soluble anti-CD28 (1 μg ml⁻¹) for 3 days, rested overnight and stimulated with IL-21 (100 ng ml⁻¹) for 1 h (ChIP-Seq) or 4 h (EMSA). For T\(_{17}\) polarization, cells were subjected to 2 rounds of polarization with anti-CD3/anti-CD28 for 4 days in the presence of IL-6 (10 ng ml⁻¹), TGFB (2 ng ml⁻¹), anti-IFN-gamma (10 μg ml⁻¹), and anti-IL-4 (10 μg ml⁻¹). Unlike the CD4⁺ T cells, T\(_{17}\) cells were not stimulated with IL-21.

Mice. Wild-type, Batf\(^{-/-}\) and Irf4\(^{-/-}\) mice were 6–8 weeks old C57BL/6 back-ground mice of mixed gender. All experiments with mice were performed under protocols approved by the NHLBI Animal Care and Use Committee, and followed NIH guidelines for use of animals in intramural research.

ChIP-Seq experiments. We used chromatin from approximately 2 × 10\(^7\) cells, which corresponds to approximately 100 ng of DNA, for each ChIP-Seq library and antibodies to Irf4 (Santa Cruz, sc-6059), STAT3 (Invitrogen), BATF, JUN (Abcam, ab31419), JUNB (Santa Cruz, sc-73), and JUND (Santa Cruz, sc-74). The ChIPed DNA fragments were blunt-ended, ligated to adaptors, and sequenced using an Illumina 1/2G Genome Analyzer and HiSeq2000 platform to obtain reads of 25–50 bp, depending on the platform. Sequenced reads were aligned to the mouse genome (NCBI36/mm8, February 2006 assembly) with Bowtie 0.12.431; only uniquely mapped reads were retained. Uniquely mapped reads and non-redundant reads numbers for each library are listed in Supplementary Table 2.

The output of Bowtie was converted to BED files, which represent the genomic coordinates of each read. Reads were mapped into non-overlapping 200-bp windows, and the location of reads on positive (negative) strand was shifted ± 75 bp from its 5’ start to determine the approximate centre of the DNA fragment associated with the reads. With these locations, the reads in each 200-bp summary window were counted. BEDGraph files were generated and viewed using the UCSC genome browser, and we aligned the BATF, IRF4, JUN and STAT3 binding sites in the UCSC genome browser; 24,769 genes were used for RNA-Seq analysis and Database of genes.

Motif discovery. The motifs of interest were discovered in B cells, T cells, and TH17 cells. Motif discovery was also performed for other transcription factors, including BATF, STAT3, JUN, JUNB and JUND. Where indicated, the five most significant motifs are shown; motifs were sorted by consensus E-values or by motif occurring frequencies.

De novo motif discovery. Owing to the computational complexity, for each library we selected the top 1,000 peaks with lowest P values, extracted 100 bp of DNA sequence centred on the ‘summit’ for each peak, and performed de novo motif analysis using MEME\(^5\) to characterize the IRF4/IRF8 consensus binding motifs in B cells, T cells, as well as T\(_{17}\) cells. Motif discovery was also performed for other transcription factors, including BATF, STAT3, JUN, JUND and JUND. Where indicated, the five most significant motifs are shown; motifs were sorted by consensus E-values or by motif occurring frequencies.

Identification of binding sites. ChIP-Seq experiments were performed to identify transcription factor binding sites in splenic B cells, CD4⁺ T cells and T\(_{17}\) cells. We used MACS 1.3.7.1\(^9\) to call binding sites (peaks) relative to a control IgG library as input control. The P-value threshold was set as 1 × 10\(^{-10}\). To call a peak, the total number of reads in each peak region need to be > 20 with FDR < 0.1. Only non-redundant reads were analysed for peak calling.

Genome-wide distribution analysis. To call a peak, the total number of reads in each peak region need to be > 20 with FDR < 0.1. Only non-redundant reads were analysed for peak calling.

De novo motif discovery. Owing to the computational complexity, for each library we selected the top 1,000 peaks with lowest P values, extracted 100 bp of DNA sequence centred on the ‘summit’ for each peak, and performed de novo motif analysis using MEME\(^5\) to characterize the IRF4/IRF8 consensus binding motifs in B cells, T cells, as well as T\(_{17}\) cells. Motif discovery was also performed for other transcription factors, including BATF, STAT3, JUN, JUND and JUND. Where indicated, the five most significant motifs are shown; motifs were sorted by consensus E-values or by motif occurring frequencies.

Motif scanning analysis. For the motif scanning analysis related to Fig. 1g, AP1 motifs from Fig. 1d were centred and 100 bp of DNA sequence 5’ and 3’ were analysed for the proximal IRF motif, 5’-GAAA/T/TTC-3’. Matched motif hits were counted at each nucleotide position and then plotted using a histogram, with breaks set at 200.

Genome-wide distribution analysis. The 5’ UTR, 3’ UTR, introns, exons and intergenic regions were defined according to the RefSeq database. Promoter regions were defined as regions extending 15 kb 5’ of the transcription start site. Peaks up to 5 kb 3’ of the transcription end site were considered as binding within the gene body.

Reporter assays. CD4⁺ T cells were activated for 24 h with anti-CD3 + anti-CD28, washed, rested overnight, and 10⁷ cells electroporated with 20 μg reporter plasmid and 1 μg pRLTK in 0.2 ml RPMI using 960 μF and 250 V. Cells were immediately stimulated with IL-21, anti-CD3/anti-CD28, or IL-21 + anti-CD3/anti-CD28. Dual luciferase assays were performed 7 h later (Promega). Shown is luciferase activity relative to the control pRLTK activity.

Electrophoretic mobility shift assays. Nuclear extracts were prepared as described\(^4\). Binding reactions contained 5 μg extract, 1.5 μg poly-deoxynucleosine-deoxyctydyl acid (poly dI:dC), and 30,000 c.p.m. of 32P-labelled probe. For supershift analysis, extracts were pre-incubated for 20 min on ice with antibodies to IRF4 (M-17), JUNB (N-17), JUND (329), BATF (WW8), FOS (4), FRA2 (H103), PU.1 (T-21) (Santa Cruz Biotechnologies). Reactions were electrophoresed to 32% polyacrylamide gels in 0.5× TBE buffer.

10. Langmead, B., Trapnell, C., Pop, M. & Salzberg, S. L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10, R25 (2009).
11. Zhang, Y. et al. Model-based analysis of ChIP-Seq (MACS). Genome Biol. 9, R137 (2008).
12. Bailey, T. L. & Elkan, C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proc. Int. Conf. Intell. Syst. Mol. Biol. 2, 28–36 (1994).