Technical Manual **Screening**

Dharmacon™ NGS Library Prep Kit

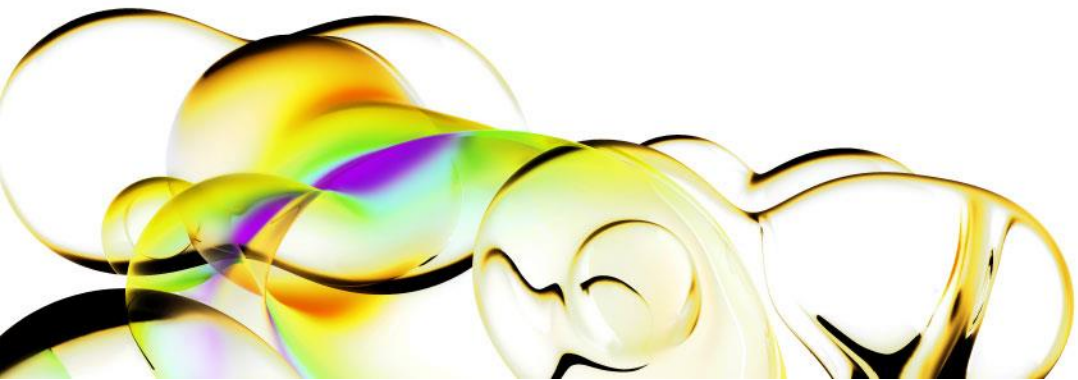
Storage : -20°C

Kit Contains: 12 or 24 BARCODES | 12 or 24 whole genome screens

Version: 0001 **Revision date:** 052025

For research only. Not for use in diagnostic procedures.

Horizon Discovery Biosciences Ltd. (A Revvity Company)



Contents

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1 General information

A. Product overview

The NGS Library Prep Kits are used in combination with Dharmacon™ lentiviral pooled screening libraries and are designed to amplify and sequence sgRNA constructs from gDNA without bias, such that differences in sgRNA representation after sequencing are due to enrichment or depletion that occurs during the primary screen.

There are two main steps involved in Dharmacon pooled library amplicon processing. First, sgRNA constructs within the isolated gDNA are amplified using vector-specific NGS Hit Identification Primers that have been designed and optimized to minimize amplification bias and introduce diversity into the high-throughput sequencing run. A second PCR amplification integrates relevant flow cell binding domains and unique 12 base pair sample indices. A limited number of cleanup steps ensures maximum recovery of amplicons for downstream sequencing. These libraries can then be multiplexed and sequenced on an Illumina® platform. Custom sequencing read primers are not required.

Please follow the manufacturer's instructions for Illumina platform sequencing. For accurate hit identification, we recommend obtaining a minimum of 1000 1 × 150 bp reads per sgRNA. If the output of your sequencing platform is higher than the number of reads required per sample, it may be possible to multiplex samples in the same run. To calculate the maximum number of samples that can be loaded per lane, it is advisable to assume a lower number of sequence reads than the maximum obtained on an optimum sequencing run. We suggest estimating the number of reads by adjusting the manufacturer's specifications by a factor of 0.7.

B. Kit Overview

The Dharmacon™ NGS Library Prep Kits contain enough material to prepare 12 or 24 samples from whole genome (WG) screens performed with 4 sgRNAs/ gene at 200-fold representation/ sgRNA for Illumina® compatible sequencing. Fewer samples can be prepared from WG screens performed with 8 sgRNAs/ gene or at >200-fold representation. Please refer to **Table 1** and either **Appendix A** or the Pooled sgRNA Screening Protocol Tracking Worksheet to determine the number of PCR I reactions required for your experiment.

Note: The 12 WG screen kit contains PCR II Primers 97-108, the 24 WG screen kit contains PCR II Primers 97-120.

C. Contents, storage and shelf life

The shelf life of all reagents is at least 12 months when stored properly.

The NEXTFLEX® NGS Cleanup Beads should be stored at 4°C, and all other components should be stored at -20°C.

Kit Contents	Amount (12 or 24 WG screens)
NEXTFLEX® PCR Master Mix (green cap)	3 x 1200 µL/ 6 x 1200 µL
Dharmacon HIT Identification Primers hEF1α or mCMV (orange cap)	300 µL/ 600 µL
NEXTFLEX® PCR II Barcoded Primer Mix (plate)	4 µL
Resuspension Buffer	12 mL/ 24 mL
Nuclease-free Water	8 mL/ 16 mL
NEXTFLEX® NGS Cleanup Beads	7 mL/ 15 mL

Table 1. Contents of 12 or 24 WG screen NGS Library Prep Kits.

D. Required materials not provided

- High-quality genomic DNA in nuclease-free water (See **Appendix A** to determine the amount of gDNA needed per sample)
- 96 well PCR plate non-skirted (Phenix Research, Cat # MPS-499) or similar

- Adhesive PCR Plate Seal (BioRad, Cat # MSB1001)
- Magnetic Stand -16 (Invitrogen™, Cat # 12321D), -96 (Ambion, Cat # AM10027) or similar
- Thermocycler
- 2, 10, 20, 200 and 1000 µL pipettes / multichannel pipettes
- Nuclease-free barrier pipette tips
- Vortex
- 80% Ethanol, freshly prepared (room temperature)

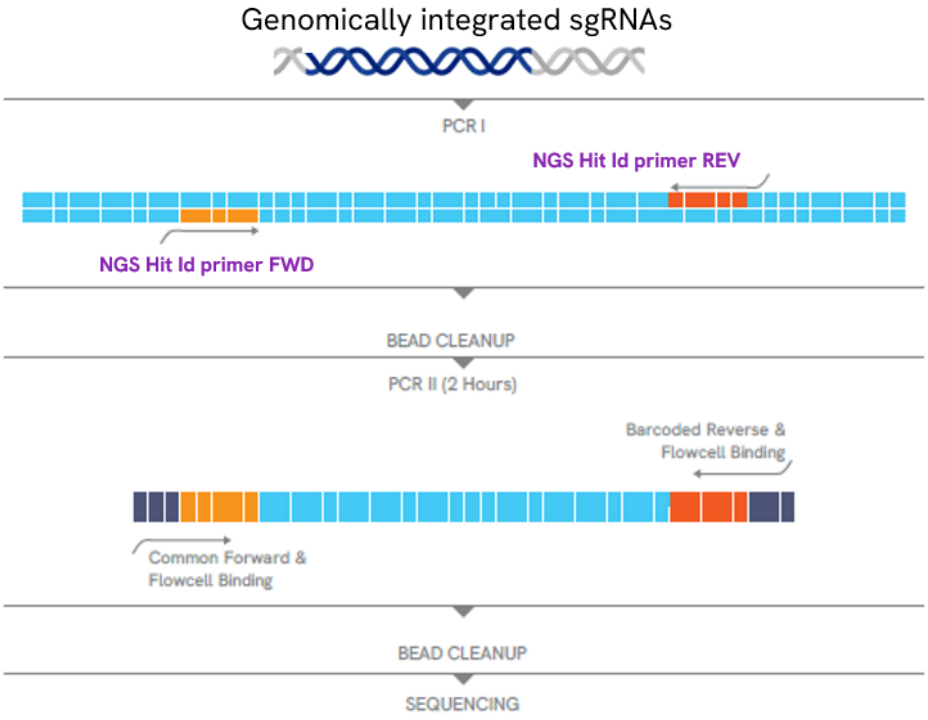
E. Warnings and precautions

Revvity strongly recommends that you read the following warnings and precautions. Periodically, optimizations and revisions are made to the components and manual. Therefore, it is important to follow the protocol included with the kit. If you need further assistance, you may contact your local distributor or Revvity at ngs@revvity.com or technical@horizondiscovery.com (North America and Europe).

- Do not use the kit past the expiration date.
- Once the plate containing the NEXTFLEX® PCR II Barcoded Primer mix has thawed, spin for one minute before use. This is to ensure all liquid settles to the bottom of the plate.
- The plate seal is intended to be pierced. Do not peel the plate seal from the plate, doing so can easily lead to cross-contamination. Additional thermal heat seals may be applied upon one another to re-seal plate.
- Before use, carefully mix PCR II Barcoded Primers by pipetting up and down several times using a multi- channel pipette with barrier tip. NEVER mix plates by vortexing. Placing a plate on a vortexer to mix samples or barcodes has been proven to result in cross-contamination, even if the plate appears to be securely sealed.
- Ensure pipettes are properly calibrated as library preparations are highly sensitive to pipetting error.
- Try to maintain a laboratory temperature of 20º–25ºC (68º–77ºF).
- Genomic DNA sample quality may vary between preparations. It is the user's responsibility to utilize high quality Genomic DNA. Genomic DNA that is heavily nicked or damaged may cause library preparation failure. Absorbance measurements at 260 nm are commonly used to quantify DNA and 260 nm / 280 nm ratios of 1.8 usually indicate relatively pure DNA. Other quantification methods using fluorescent dyes may also be used. The user should be aware that contaminating RNA, nucleotides and single-stranded DNA may affect the amount of usable DNA in sample preparation.
- It is required that Dharmacon HIT Identification Primers (hEF1α or mCMV) & NEXTFLEX® PCR II Barcoded Primer Mix are used during PCR amplification steps.

2 Preparation protocol

A. Sample preparation flow chart



B. Starting material

The Dharmacon™ NGS Library Prep Kits have been optimized and validated using 5 µg of high-quality genomic DNA per 50 µL reaction. NGS analysis of samples from pooled screens will often require > 5 µg of gDNA to maintain a minimum of 200-fold representation/ sgRNA, thereby necessitating multiple PCR I reactions. These reactions can be combined following PCR I cleanup.

Please refer to **Appendix A** or the Pooled sgRNA Screening Protocol Tracking Worksheet to calculate the amount of gDNA and number of PCR I reactions needed prior to starting your experiment.

C. Reagent preparation

- Briefly spin down each component to ensure material has not lodged in the cap or side of tube. Keep on ice and vortex each NEXTFLEX® Mix just prior to use.
- Allow NEXTFLEX® NGS Cleanup Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.
- **Note**: Due to the viscosity of certain materials, attempting to prepare more than the stated number of reactions may result in a shortage of materials. All NEXTFLEX® enzyme components must be centrifuged at 600xg for 5 seconds before opening the tube(s).

STEP A: PCR I Amplification

Revvity supplied materials:

- NEXTFLEX® PCR Master Mix
- Dharmacon HIT Identification Primers (hEF1α or mCMV)
- Nuclease-free water

User supplied materials:

- Thermocycler

- 96 well PCR plate
- High-quality genomic DNA in nuclease-free water

1. For each sample, combine the following reagents on ice. **Note**: many screens will require multiple PCR I reactions per sample to maintain adequate representation.

Component	Volume per reaction (µL)
High-quality genomic DNA (up to 5 µg/RXN)	-
Nuclease-free water	-
NEXTFLEX® PCR Master Mix **(green cap)**	12
Dharmacon HIT Identification Primers hEF1α or mCMV (orange cap)	1
TOTAL	**50 µL**

2. Mix well by pipetting.

3. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

Temperature	Time	
98 °C	4 minutes	
98 °C	30 seconds	**17-20 Cycles**
58 °C	30 seconds	
72 °C	30 seconds	
72 °C	4 minutes	

STEP B: PCR I Cleanup

Revvity supplied materials:

- Resuspension Buffer
- NEXTFLEX® NGS Cleanup Beads

User supplied materials:

- 80% Ethanol, freshly prepared (room temperature)
- Magnetic Stand

1. Add 16 µL of NEXTFLEX® NGS Cleanup Beads and 34 µL of nuclease free water to each 50 µL reaction. Mix thoroughly by pipetting. The NEXTFLEX® NGS Cleanup Beads and Nuclease-free water can be premixed and added in a single step.

2. Incubate at room temperature for 5 minutes.

3. Place on the magnetic stand at room temperature until the supernatant appears completely clear.

4. Remove and discard the supernatant. Do not disturb beads. Some liquid may remain in wells.

5. While on magnetic stand, add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate at room temperature for 30 seconds. Carefully remove ethanol by pipette.

6. Repeat previous step, for a total of 2 ethanol washes. Ensure all ethanol has been removed.

7. Remove from the magnetic stand and let dry at room temperature for 3 minutes.

8. Resuspend dried beads with 22 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.

9. Incubate resuspended beads at room temperature for 2 minutes.

10. Place plate on magnetic stand for 5 minutes or until the sample appears clear.

11. Transfer 20 µL of clear supernatant (purified PCR I product) to a 1.7 mL tube. **Combine all reactions from a given sample in a single tube and mix thoroughly.**

STEP C: PCR II Amplification

Revvity supplied materials:

- NEXTFLEX® PCR Master Mix
- NEXTFLEX® PCR II Barcoded Primer Mix

User supplied materials:

- Thermocycler
- 96 well PCR plate
- Purified PCR I product (from STEP B)

1. Vortex purified PCR I product for >10 seconds to ensure homogeneity. **Note**: a single PCR II reaction is sufficient to prepare a sample from a WG screen performed with 4 sgRNAs/ gene.

2. For each sample, combine the following reagents on ice. **Note**: make sure to spin down the plate containing the PCR II Barcoded Primers prior to opening and mix by pipetting. NEVER mix plates by vortexing.

Component	Volume per reaction (µL)
Purified PCR I product (from STEP B)	36
NEXTFLEX® PCR Master Mix **(green cap)**	12
NEXTFLEX® PCR II Barcoded Primer Mix (plate)	2
TOTAL	**50 µL**

3. Mix well by pipetting.

4. Apply adhesive PCR plate seal and place in thermocycler for the following PCR cycles:

Temperature	Time	
98 °C	4 minutes	
98 °C	30 seconds	**10-12 Cycles**
60 °C	30 seconds	
72 °C	30 seconds	
72 °C	4 minutes	

STEP D: PCR II Cleanup and library validation

Revvity supplied materials:

- Resuspension Buffer
- NEXTFLEX® NGS Cleanup Beads

User supplied materials:

- 80% Ethanol, freshly prepared (room temperature)
- Magnetic Stand

1. Add 45 µL of NEXTFLEX® NGS Cleanup Beads to each 50 µL reaction. Mix thoroughly by pipetting.

2. Incubate at room temperature for 5 minutes.

3. Place on the magnetic stand at room temperature until the supernatant appears completely clear.

4. Remove and discard the supernatant. Do not disturb beads. Some liquid may remain in wells.

5. While on magnetic stand, add 200 µL of freshly prepared 80% ethanol to each magnetic bead pellet and incubate at room temperature for 30 seconds. Carefully remove ethanol by pipette.

6. Repeat previous step, for a total of 2 ethanol washes. Ensure all ethanol has been removed.

7. Remove from the magnetic stand and let dry at room temperature for 3 minutes.

8. Resuspend dried beads with 22 µL of Resuspension Buffer. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.

9. Incubate resuspended beads at room temperature for 2 minutes.

10. Place plate on magnetic stand for 5 minutes or until the sample appears clear.

11. Transfer 20 µL of clear supernatant (purified PCR I product) to a new well or tube.

12. To ensure cluster generation, it is recommended that you quantify your library by gel or fragment analysis. To quantify by gel, load 2 µL of 6X Gel Loading Dye and 6-10 µL of PCR Product on a 2% low melt agarose gel + SYBR Gold.

Amplicons from screens performed with libraries using the hEF1α promoter will be 309-318 bp in size; those from screens performed with libraries using the mCMV promoter, including Edit-R Lentiviral sgRNA Pooled Libraries, will be 391-400 bp in size.

Note that the staggered diversity spacers in the Dharmacon HIT Identification Primers will generate ten roughly equimolar amplicons varying by 1 bp in length.

13. Quantitate DNA library templates for optimal cluster density. Libraries generated with this kit are sequenced with standard Illumina sequencing primers with minimum of 1000 1 × 150 bp reads per sgRNA.

3 Appendix

A. Number of PCR reactions

Calculate the minimum amount of gDNA required to maintain the desired sgRNA fold representation, assuming a single lentiviral integration event per cell (genome), using the following formula:

Number of cells with lentiviral integrations **×** 6.6×10^{-3} ng/ genome (see note below and Appendix B) **=**

The calculation above assumes a diploid human genome. For cells that are not diploid, calculations should be adjusted accordingly.

We have optimized the PCR conditions to remain in the linear phase of log amplification. We recommend a maximum of 5 µg of gDNA per 50 µL PCR RXN; using more gDNA per PCR could inhibit the efficiency of the reaction and may result in biased amplification.

Calculate the number of PCRs required for each sample using the following formula:

Mass of gDNA required to maintain representation of each sgRNA (ng) **÷** 5000 ng per reaction **=**

* Round up the value obtained to the next whole number

The calculations above are used to determine the number of PCR reactions required per sample. To determine the total number of PCR reactions required for your screen use the following formula:

Number of PCR reactions per sample × Number of samples =

Calculation example

If you anticipate 1.52×10^7 cells with lentiviral integrations, the mass of gDNA required would be calculated as follows:

1.52×10^7 lentiviral integrations × 6.6×10^{-3} ng/genome = 1×10^5 ng

The maximum amount of gDNA per PCR is 500 ng, therefore the number of PCR reactions necessary can be calculated as follows:

1×10^5 ng gDNA ÷ 5000 ng/PCR = 20PCR reactions

If you have four samples (reference and experimental samples in biological duplicates), then you need the following total number of PCR reactions:

20 PCR reactions × 4 samples = 80 PCR reactions

B. Calculating the mass of the human genome

The mass of the human genome can be calculated using the below values.

Constants	Value
Mass of a base pair	**660 g/mol/bp**
Base pairs per diploid human genome	**6×10^9 bp**
Avogadro constant	**6.02×10^{23} mol^{-1}**

Calculation example

Calculate the mass of human genome:

$(6 \times 10^9$ bp/genome) × (660 g/mol/bp) = 4×10^{12} g/mol/genome

$(4 \times 10^{12}$ g/mol/genome) ÷ (6.02×10^{23} mol -1) = 6.6×10^{-12} g/genome

C. Oligonucleotide sequences

Dharmacon HIT Identification Primers (hEF1α or mCMV)	Sequence 5' → 3'
Forward primer extension*	ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Reverse primer extension	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

*Note that the forward primers contain a stagger site between the 5' Illumina extension and the priming site to introduce nucleotide diversity during sequencing, which will need to be adjusted for when the sequences are trimmed. For more information, please see our Bioinformatic Analysis of Dharmacon™ Lentiviral sgRNA Pooled Library Screens Protocol. For priming site sequences, please contact our Scientific Support team.

NEXTFLEX® PCR II Barcoded Primer Mix	Sequence 5' → 3'
PCR II Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACG CTCTTCCGATCT
PCR II Reverse	CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXX[1]GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

[1]XXXXXXXXXXXX denotes the index region of adapter. The index sequences and the respective reverse complement sequences contained in each adapter are listed below.

D. Reverse primer index sequences and reverse complements

Barcoded primer	Index sequence (5' → 3')	Reverse complement
97	GGATGGTACGCA	TGCGTACCATCC
98	CCGAACCGGCTT	AAGCCGGTTCGG
99	GGATGCAGTTAT	ATAACTGCATCC
100	CCGAAGGCCCTC	GAGGGCCTTCGG
101	GGCTGCACAATA	TATTGTGCAGCC
102	CCGAAGGTTTCT	AGAAACCTTCGG
103	GGATGCATGGCG	CGCCATGCATCC
104	CCGAAGGAAAGA	TCTTTCCTTCGG
105	GGATGCAACCGC	GCGGTTGCATCC
106	AAGAATTGGGAT	ATCCCAATTCTT
107	GGCTGTGGTCGA	TCGACCACAGCC
108	AAGAACCAAGAG	CTCTTGGTTCTT
109	GGCTGTGCAGCT	AGCTGCACAGCC
110	AAGAACCGGAGA	TCTCCGGTTCTT
111	GGCTGTGACTAG	CTAGTCACAGCC
112	AAGAACCTTCTC	GAGAAGGTTCTT
113	GGATGACCACGG	CCGTGGTCATCC
114	CCGAATTGGTCA	TGACCAATTCGG
115	GGATGACTGTAA	TTACAGTCATCC
116	CCGAATTAACTG	CAGTTAATTCGG
117	GGCTGACACATT	AATGTGTCAGCC
118	AAGAAGGTTGAA	TTCAACCTTCTT
119	GGATCGAGAAGC	GCTTCTCGATCC
120	AAGATATATTAT	ATAATATATCTT

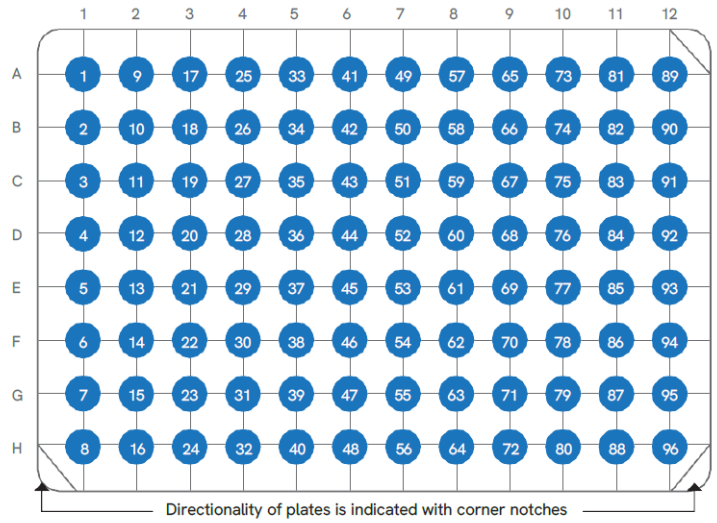
E. Low level multiplexing

Every combination of sequential odd and even numbered barcodes are fully color balanced at all positions of the index.
For example, barcodes 99 and 100 are opposite colors at every position, but barcodes 100 and 101 are not.

F. Plate format

Plate; 4 µL/well

Representative plate orientation:



12 WG screen kits contain only PCR II Primers 97-108 arrayed in columns 1-2

24 WG screen kits contain only PCR II Primers 97-120 arrayed in columns 1-3

For questions concerning the design or production of the products, please contact our Scientific Support team.

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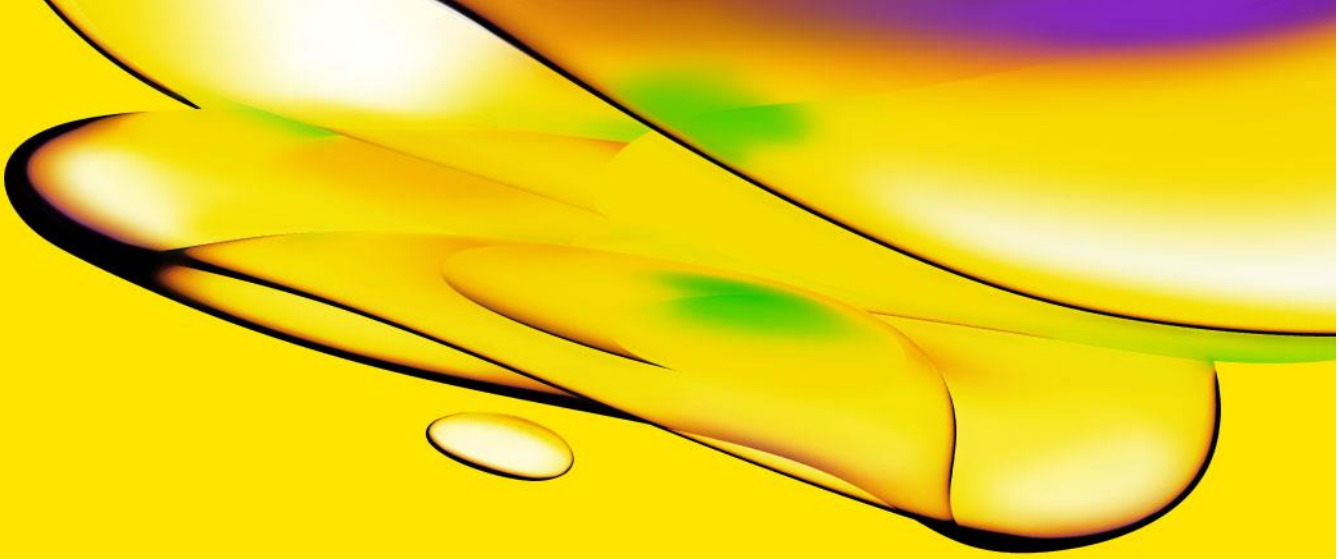

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