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EliGene® Adaptor-IL NGS

REF 90069-NGS (for 96 samples)

Kit components:

3 x 700 µl **Adaptor-IL Mix**
1 x 40 µl **p701 MID Primer**
1 x 40 µl **p702 MID Primer**
1 x 40 µl **p703 MID Primer**
1 x 40 µl **p704 MID Primer**
1 x 40 µl **p705 MID Primer**
1 x 40 µl **p706 MID Primer**
1 x 40 µl **p707 MID Primer**
1 x 40 µl **p708 MID Primer**
Instruction for use
1 x 40 µl **p501 MID Primer**
1 x 40 µl **p502 MID Primer**
1 x 40 µl **p503 MID Primer**
1 x 40 µl **p504 MID Primer**
1 x 40 µl **p505 MID Primer**
1 x 40 µl **p506 MID Primer**
1 x 30 µl **RP1 Primer**
1 x 30 µl **RP2 Primer**
1 x 30 µl **Index Primer**

Storage and shelf life after first opening:

All components of the kit must be transported and stored at -20 °C. Kit and remaining MasterMixes must be stored at -20 °C in a dark.

Intended use

EliGene® Adaptor-IL NGS kit is intended for the incorporation of molecular identifiers (MIDs) and specific sequencing adaptors to all amplicons generated using the multiplex PCR based on EliGene® NGS kits, enabling the identification of polymorphisms in the target area.

Principle of the method

EliGene® NGS kits enable multiplex PCR amplification of all required target regions of the gene(s) of interest. The recommended amount of DNA for each multiplex PCR reaction is between 20 and 100 ng of DNA derived from formalin-fixed paraffin-embedded (FFPE) material or from blood. Next, the resulting amplicons are barcoded, and mixed per sample. Finally, the resulting amplicon libraries of different samples are pooled and sequenced using a NGS instrument Illumina for massively parallel sequencing (MPS) according to the manufacturer's instructions. The resulting sequence reads are subsequently analyzed by comparison with the reference sequence of the targeted gene(s) to identify variant positions.

Introduction

EliGene® NGS kits are used for one or more multiplex PCR reactions resulting in amplification of target regions of one sample (Figure 1a). For amplification is used a ready-to-use mastermix containing a hot-start DNA polymerase. The resulting amplicons of each multiplex are diluted 1,000 fold. In the second step, a second round of PCR is performed using EliGene® Adaptor-IL NGS kit, enabling tagging of all the amplicons to incorporate MIDs and p7 and p5 adaptors required for Illumina MiSeq Sequencing (Figure 1b). The resulting tagged amplicons from are mixed per individual applying a predefined assay specific mixing scheme. Each amplicon library is subsequently purified from small residual DNA fragments shorter than 150bp and the DNA concentration is determined using a Real-Time PCR with concentration standards. Next, these purified and individually tagged amplicon libraries are pooled equimolar, resulting in an amplicon pool or sequencing sample, which is then further processed using custom sequencing primers from EliGene® Adaptor-IL NGS kit and sequenced on the Illumina MiSeq. During the sequencing run, different sequence reads are generated on the same cluster in the process of bridge PCR. The positions of the individual custom sequencing primers supplied by EliGene® NGS kits as well as the Illumina sequencing reagents are indicated and clarified in Figure 1c.

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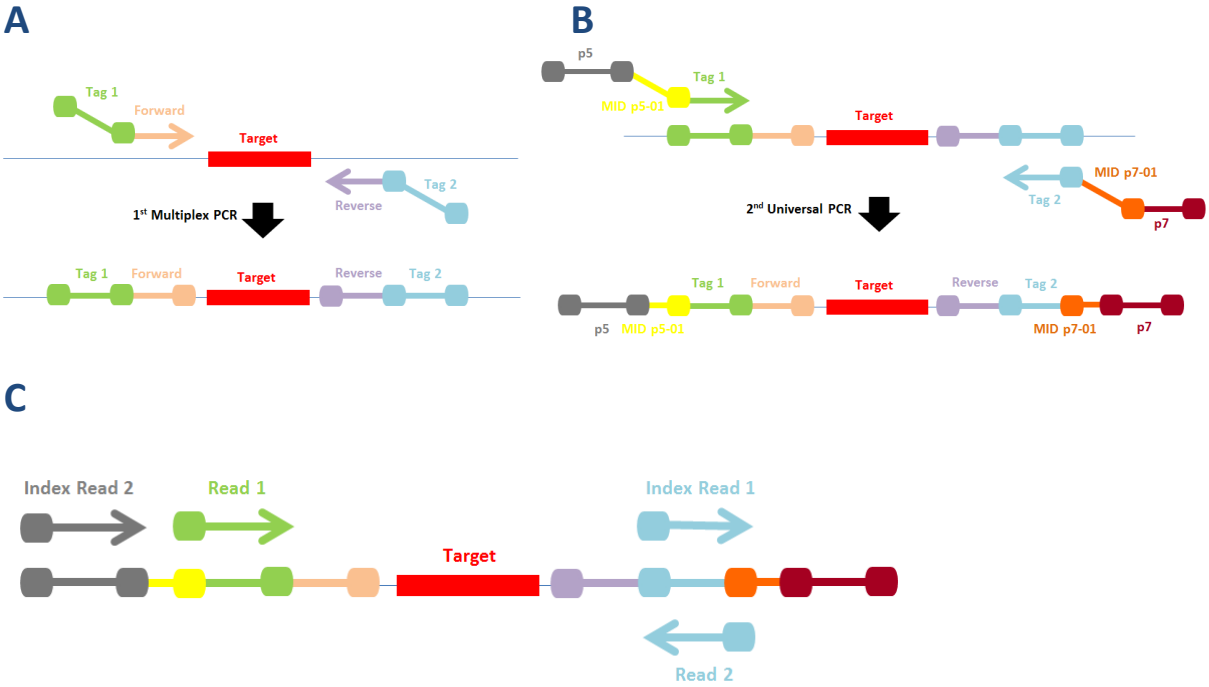

Figure 1. *Layout of the process resulting in multiplex PCR of target regions (A), incorporation of MIDs and p7 and p5 adaptors (B) and structure of resulting product after the sequencing on Illumina MiSeq instrument (C)*

Specimen

Suitable specimens are the amplicons from multiplex PCR using EliGene® Lung NGS, EliGene® Colorectum NGS and EliGene® GIST NGS kits.

It is important to avoid storage of the multiplex PCR derived amplicons at 4°C. Continue immediately with workflow, otherwise immediately store in a freezer at -20°C.

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Material and equipment required but not provided

Equipment	Recommendations/Comments
Sequencing Instrument and associated reagents for sequencing	Illumina MiSeq
Analysis software for read counts and variant calling of the generated sequencing data	BaseSpace, MiSeq Reporter, Variant Caller Studio (Illumina)
Illumina sequencing reagents	MiSeq Reagent Nano Kit, v2 (500 cycles), MiSeq Reagent Kit v2 (500cycle), MiSeq® Reagent Kit v3 (600 cycle)
TE Buffer	10 mM TRIS, 1 mM EDTA, pH=8.0
Thermal cycler	With a ramp rate of 1°C/s, temperature accuracy: ± 0.3°C (50-95°C), temperature non-uniformity across all zones < 0.5°C.
Real-time PCR cycler	With SYBR Green detection.
Quantitative PCR (qPCR) kit for Illumina library	Elisabeth Pharmacon, EliZyme™ Library Quantification Kit
Agarose gel or sensitive digital alternative	2% agarose gel, 1 x TBE buffer, DNA dye, electrophoresis bath or Agilent 2100 Bioanalyzer with Agilent High Sensitivity DNA Kit
Hi-Di Formamide	Life Technologies cat. no. 4311320
GS600 size standard	Life Technologies (e.g. GS600 LIZ®, cat. no. 4408399)
DNA fragment analyzer	Capillary Genetic analyzer with GeneMapper software (Life Technologies)
GeneRead Size Selection Kit (50)	Qiagen, cat. no. 180514
Vortex	
Centrifuge(s)	1.5 ml tubes, 12 000 x g
Sterile automatic pipette 5–20 µl and disposable tips	Sterile-packed, aerosol-resistant disposable pipette tips, DNA/RNA free, DNase/RNase free
Plastic material (strips, plates, tubes)	Sterile, DNase/RNase free
Sterile rack	DNA/RNA free, DNase/RNase free
Laboratory safety gloves	

Preparation of reagents

- To avoid the contamination keep all tubes closed and follow the instructions.
- All reagents have to be completely thawed, vortexed and centrifuged before use.

Preparation of master reaction mix

Warning: The following procedure should be strictly followed and performed by qualified personnel; any deviation from the prescribed protocol can lead to erroneous results.

Reagents in this kit are set to incorporation of specific MID sequences and P5, P7 adaptors which are necessary

for the sequencing run on MiSeq instrument. For each sample (including the negative control sample) to be sequenced in the same sequencing run on an MiSeq instrument, a master reaction mix using a unique combination of MID p7-p5 Primer Mixes should be freshly prepared. To avoid issues with low nucleotide diversity in the Illumina MiSeq run, use the Support tools in section NGS kits at www.elisabeth.cz

If you want to detect variants with an allele frequency of 10% or lower, run-to-run sample carryover can be a confounding factor. To prevent this, we recommend avoiding sequencing with the same MID combination in two consecutive runs

1. Remove the required Universal PCR Mix, MID p7 and p5 Primer Mixes from the freezer and allow complete thawing.
2. After thawing, vortex each vial thoroughly and centrifuge before use.
3. Prepare PCR mix for each combination of MID primers according to table below.

PCR Mix	MID p7 Primer	MID p5 Primer
20.0 µl	2.0 µl	2.0 µl

4. Add 1 ul of PCR product from previous multiplex PCR, diluted 1:100 with PCR water to the amplification mix. It is recommended to use two-step serial dilution (e.g. mix 2 µl of PCR product with PCR water, vortex and spin down, mix 2 µl of the dilution with PCR water, vortex and spin down).
5. Vortex and spin down the resulting PCR reaction mix and run the following thermal cycling profile:

PCR cycling profile:

95°C	3 min	
95°C	15 s	
60°C	20 s	20x
72°C	60 s	
72°C	5 min	

Set the ramp rate of the PCR machine between 1 and 2°C/s

Do not store the amplified products at 4°C: continue immediately with the workflow, otherwise immediately store amplified products at -20°C.

Library purification

Purify each amplicon library from short DNA fragments using GeneRead Size Selection Kit (Qiagen).

1. Mix 25 µl of amplifikacation product with 100 µl of SB1 buffer, vertex briefly and spin down.
2. Transfer the mixture to MinElute spin column a centrifuge for 1 min at 12000 to 14000 x g.
3. Pour out the flow through, dispense 700 µl 80% ethanol to each column and centrifuge again for 1 min at 12000 to 14000 x g.
4. Repeat step 3.
5. Pour out the flow through and centrifuge an empty MinElute spin column for 1 min at 12000 to 14000 x g.
6. Place MinElute spin column into new 1.5 ml tube and add 17 µl of EB buffer to the middle of the membrane. Let the column standing on the table for 1 minute.

7. Centrifuge the column for 1 min at 12000 to 14000 x g.

Concentration measurement of the amplicon libraries

1. Determine the concentration of the obtained purified amplicon libraries by qPCR (e.g. KAPA Library Quant Kits, KAPA Biosystems) following the instructions of use. Amplicon´s average size for MasterMixes of the EliGene® NGS kits are for COLORECTUM NGS Mix 410 bp, LUNG NGS Mix 390 bp, c-Kit NGS Mix 390 bp and for PDGFR NGS Mix 380 bp.
2. Dilute each amplicon library with measured concentration to 4 nM in 1 x TE buffer.
3. Vortex briefly and spin down.
4. If possible, continue with the following steps of the procedure.

Pooling of multiple amplicon libraries

- Take care to pool only amplicon libraries that contain unique MID combinations.
- It is highly recommended to obtain a certain minimal coverage (number of read pairs) per individual sample. Nevertheless, higher coverage may be required to detect low frequency variants.
- For somatic mutations: the tumor tissue content (TTC) of the origin FFPE material should be taken into account. A diluted amplicon library derived from a sample with lower TTC needs a higher contribution to the amplicon pool (e.g. when libraries derived from samples with a TTC of 80% and 40% respectively, a 2-fold higher volume of the latter diluted amplicon library should be pooled into the amplicon pool compared to the first library).
- Pool the amplicon libraries, vortex briefly and centrifuge.
- Proceed immediately with next step, otherwise immediately store the amplicon pool in a freezer between at -20°C.
- For the calculation of the number of samples per run, take into account that the PhiX control library added to your sample also uses part of the capacity of your run. The targeted PhiX control should have concentration of 5% according to the manufacturer advice.

Bridge PCR and sequencing

To reduce carryover, it is recommended to perform a maintenance wash prior to each sequencing run.

The amplicon pool is further processed using one of the reagents kits provided by Illumina, containing all necessary consumables and reagents for sequencing the purified amplicon library on the Illumina MiSeq following the guidelines described below.

- Choose the applicable Illumina Reagent Kit: v2 (500 cycles, 2x 250 reads), or v3 (600 cycles, 2x 300 reads) and set the number of Read1 a Read2 cycles at 200.
- Custom sequencing primers and a custom index primer are required for MiSeq based sequencing. These 3 primers are provided as a 50 µM solution and can be found in the amplification box of the MID kit for Illumina MiSeq Sequencing. The 3 Primer Mixes are termed:

  RP1 - Read1 Seq Primer

  Index - Index Seq Primer

  RP2 - Read2 Seq Primer
- From each Primer Mix, prepare 600 µl of custom primer at 0.5 µM by adding 6 µl of the Primer Mix to 594 µl of HT1 (Hybridization Buffer). Mix well by vortexing and spin down.

- Load 600 µl of custom primer in the appropriate port on the cartridge:

  Read1 seq Primer to cartridge position 18

  Index Seq Primer to cartridge position 19

  Read2 seq Primer to cartridge position 20
- Inspect the bottom of the cartridge to make sure that no bubbles are present. Remove bubbles by tapping the cartridge.
- Denature and dilute the prepared amplicon pool to the desired concentration as described in the MiSeq system user guide. We advise to start with 10 pM for MiSeq Reagent kit v2, or 13 pM for MiSeq Reagent kit v3, but this can be increased to reach the optimal cluster density for your system.
- Spike-in PhiX Control v3 (Catalog No. FC-110-3001, Illumina) as described in the MiSeq system user guide to obtain the Final Sample.
- Pipette 600 µl of the Final Sample into the Load Samples reservoir.
- Create a sample sheet with the Illumina Experiment Manager as following the guidelines below::

1) Select as Instrument “MiSeq”, click Next.
2) Select as Category “Targeted Resequencing”, select as Application “PCR Amplicon”, click Next.
3) Fill out the PCR Amplicon Run settings, with these specific settings:

   Sample Prep Kit: select “Nextera”

   o Index Reads: select “2”

   o Read Type: select “Paired End”

   o Cycles Read1: 200

   o Cycles Read2: 200

   o Keep “Use Adapter Trimming” selected
4) Change the PCR Amplicon Workflow-Specific Settings: select the 3 “Custom Primers”. Click Next.
5) Add rows to the sample sheet. Select Indexes from the list, ignore eventual warning on diversity of index cycles, and select the correct Manifest file. The correct Manifest file has to be downloaded from www.elisabeth.cz

- Proceed directly with the run setup steps using the MiSeq Control Software (MCS) interface.

Reading and interpretation of results

Data set

MiSeq instrument generates two reads for each cluster (Read 1 and Read 2), which will be defined as read pair. After the run, two index reads automatically de-multiplex the data with use of MiSeq Reporter program. The result are FASTQ files in Sample Sheet table. They include only quality filtered data from the reads.

Structure of sequencing reads

Individual reads have the following structure: Read1 begins in location with PCR forward primer and continues with the amplified region. In some cases may this sequence continue with reverse sequence of reverse primer. Read 2 has similar structure, but begins with PCR reverse primer and continues with the amplified region. In some cases may this sequence continue with sequence of forward primer. To assure correct evaluation of the results, it is necessary to trim out the used PCR primers.

Data comparison to the reference sequence

The resulting sequence reads are subsequently analyzed by comparison with the reference sequence of the targeted gene(s) of human genome. For comparison of specific information (e.g. mutation positions) included in the dataset may be different programs used, e.g. Variant Studio program from Illumina.

- Illumina® VariantStudio™ ver. 2.2.2 or higher should be used following the guidelines in the manufacturer´s manual.
- First, the program should be installed and launched from Illumina® Base Space®. Select your project and after initialization of the program select file from file list which to be added. Choose „All variants „ in next window to import variants from your VCF file.
- The KRAS, NRAS, BRAF, PDGFRAα, c-Kit and EGFR reference sequences for „Genes“ are defined in sample sheet according Table 1.

Table 1. Reference sequences for genes according to reference sequence hg19

Name	Chromosome	Amplicon Start	Amplicon End
NRAS_Ex2	chr1	115258600	115258827
NRAS_Ex3	chr1	115256394	115256693
NRAS_Ex4	chr1	115252139	115252396
KRAS_Ex2	chr12	25398151	25398344
KRAS_Ex3	chr12	25380077	25380397
KRAS_Ex4	chr12	25378457	25378790
BRAF_Ex15	chr7	140452987	140453265
PDGFRA_Ex8	chr4	55136751	55136967
PDGFRA_Ex10	chr4	55139665	55139930
PDGFRA_Ex12	chr4	55140985	55141199
PDGFRA_Ex14	chr4	55143970	55144249
PDGFRA_Ex18	chr4	55151973	55152205
CKIT_Ex9	chr4	55591944	55592236
CKIT_Ex11	chr4	55593514	55593750
CKIT_Ex13	chr4	55594120	55594371
CKIT_Ex14	chr4	55595417	55595724
CKIT_Ex15	chr4	55597418	55597689
CKIT_Ex16	chr4	55597939	55598192
CKIT_Ex17	chr4	55599207	55599372
EGFR_Ex18	chr7	55241580	55241823
EGFR_Ex19	chr7	55242332	55242535
EGFR_Ex20	chr7	55248879	55249188
EGFR_Ex21	chr7	55259388	55259645

- Click to “All Variants of Current Sample” in “Annotate” of “Annotation and Classification” option
- In „General“ option (left panel) should be select all type of „Genotypes“, all „Variant Type“ and „All Chromosomes” in „Chromosomes“ option.
- In “Variant” apply “Pass Filter” to any sample.

- After analysis verify the coverage of target positions, that is, number of reads obtained at a given position during the sequencing run. The minimum coverage required for clinical diagnostics is ≥ 100 read pairs. Only target positions with ≥ 100 read pairs are to be utilized for the final report; those below 100 read pairs should be reported as exluded regions due to insufficient coverage.

Control process

Minimal coverage and allelic frequency

For somatic mutations detection is necessary to achieve minimal coverage (number of read pairs) of 100 reads/allele. It is strongly recommended to eliminate areas with lower coverage from further analysis. In case of variant allele frequency is the minimal recommended value 25% at the minimum coverage of 100 reads/allele.

Quality score

The quality of bases comparison in the spot of detected variant has high impact on reliability of read. This quality is based on position of the variant (quality of read increases during firs 20 cycles, than stays equal until the last 20 cycles when it begins do decrease) in the read and the composition of the genome in the area of the variant (e.g. homopolymerous segments have negative influence on the quality). In case of reading from both

sides and the overlap in the reads, the passage witch overlaps has always higher Q-Score when compared to passages with one direction read. Especially cautious data analyzing should be applied in areas with homopolymerous segments longer than 10 bp.

Reference material

To control all steps of the procedure, including DNA isolation, library preparation and sequencing you may use reference material positive for selected variation in target area. There are no commercial positive controls available.

Troubleshooting

1. Errors in amplicon sequencing may be caused by a failure in library preparation, library quantification or sequencing run preparation.

2. If the library preparation was correct, the error may be caused by sequencing reagents, use of kit after expiration or a failure of MiSeq instrument.

Performance characteristics

Analytical performance characteristics:

EliGene® NGS kit is set to incorporate of adaptor sequences necessary for sequencing on MiSeq instrument. As initial material are used the amplificates from previous multiplex PCR with EliGene NGS kits.

Analytical sensitivity is 0.1 ng of DNA in reaction mix.

Analytical specificity of method is 100%. Analytical specificity of the method was validated by searching in the DNA databases.

Measuring interval

The kit enables the detection of ≥ 0.1 ng DNA molecules in reaction mix.

Internal control of quality

As an internal control of quality the amplification process of selected regions is used.

Limitation of the examination procedure

The sensitivity of kit depends on handling with specimen (isolation of DNA). It is strictly recommended to use isolation kits and procedures mentioned above.

Biological reference intervals

Not applicable information for this kit.

Warning

Unused content of the tube with MasterMix is stable for 2 weeks at -20 °C. Do not freeze tubes with MasterMix more than 5 times! Do not mix components of the kits of different lots.

Warnings and general precautions

- Handle and dispose of all biological samples as if they were capable of transmitting infective agents. Avoid direct contact with the biological samples. Avoid splashing or spraying. The materials that come into contact with biological samples must autoclaved at 121 °C for one hour before disposal.
- Handle and dispose of all reagents and all assay materials as if they were capable of transmitting infective agents. Avoid direct contact with the reagents. Avoid splashing or spraying. Waste must be treated and disposed of in compliance with the appropriate safety standards. Disposable combustible materials must be incinerated. Liquid waste containing acids or bases must be neutralized before disposal.
- Wear suitable protective clothing and gloves and protect eyes/face.
- Never pipette solutions by mouth.
- Do not eat, drink, smoke or apply cosmetic products in the work areas.
- Wash hands carefully after handling samples and reagents.
- Dispose of leftover reagents and waste in compliance with regulations in force.
- Read all the instructions provided with the kit before running the assay.
- Follow the instructions provided with the kit while running the assay.
- Do not use the kit after the expiry date.
- Only use the reagents provided in the kit and those recommended by the manufacturer.
- Do not mix reagents from different batches.
- Do not use reagents from other manufacturer´s kit.

Warnings and precautions for molecular biology

- Molecular biology procedures, such as extraction, reverse transcription, amplification and detection of nucleic acids, require qualified staff to prevent the risk of erroneous results, especially due to degradation of the nucleic acids contained in the samples or due to sample contamination by amplification products.
- It is necessary to have separate areas for the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never introduce an amplification product in the area designed for extraction/preparation of amplification reactions.
- It is necessary to have lab coats, gloves and tools which are exclusively employed in the extraction/preparation of amplification reactions and for the amplification/detection of amplification products. Never transfer lab coats, gloves or tools from the area designed for the amplification/detection of amplification products to the area designed for the extraction/preparation of the amplification reactions.
- The samples must be exclusively employed for this type of analysis. Samples must be handled under a laminar safety box. Tubes containing different samples must be never opened at the same time. Pipettes used to handle samples must be exclusively employed for this specific purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Reagents must be handled under PCR box or laminar flow box. The reagents required for amplification must be prepared in such a way that they can be used in a single session. The pipettes employed to handle the reagents must be used exclusively for this purpose. The pipettes must be of the positive displacement type or be used with aerosol filter tips. The tips employed must be sterile, free from DNases and RNases, free from DNA and RNA.
- Amplification products must be handled in such way as to reduce dispersion into the environment as much as possible, in order to avoid the possibility of contamination. Pipettes used to handle amplification products must be employed exclusively for this specific purpose.

Warnings and precautions specific to components of the kit

- The tubes containing mixes (Adaptor-IL Mix) are disposable and therefore must be used once only in the preparation of the reaction mixture.
- These mixes carry the following safety warnings (P):

P280 Wear protective gloves/protective clothing/eye protection/face protection.

P281 Use personal protective equipment as required.

Literature

De Leeneer K, De Schrijver J, Clement L, Baetens M, Lefever S, et al. (2011) Practical Tools to Implement Massive Parallel Pyrosequencing of PCR Products in Next Generation Molecular Diagnostics. PLoS ONE 6(9): e25531. doi:10.1371/journal.pone.0025531

Symbols

Catalog number


Upper limit of temperature

Batch code


Use by (last day of month).

In vitro diagnostic medical device


Fulfilling the requirements of European Directive 98\79\EC for *in vitro* diagnostic medical device.

Contains sufficient for "N" tests


Attention, consult instructions for use


Manufacturer

Manufacturer

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